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EDITOR'S NOTE:

Due to a printer's error, lines 30–31, p. 322 of the article by E. N. Harvey, entitled, "Biological Aspects of Ultrasonic Waves, A General Survey" in the December issue, were incorrectly printed. The sentence should read as follows:

". . . In one, for increasing pressure without dissolving more gas, a pyrex test-tube is sealed by deKhotinsky cement to a brass chamber connected with a pressure gage, a petcock and a screw-piston for increasing the pressure (Fig. 6A)."

In the higher organisms which, unlike the plants, have not the power to synthesize amino acids, material for the synthesis of the proteins required for growth and maintenance must be ingested in the form of proteins or amino acids. The proteins, before final absorption, must be broken down in the digestive tract into the constituent amino acids by means of enzymes, and these amino acids are built up in the body into the infinite variety of proteins characteristic of the various organs and tissues.

Now it is not difficult to conceive the possibility of building up this immense variety of different proteins from the twenty or so different amino acids presented to it. But the mechanism by which the differentiation is achieved is more difficult to imagine, and provides a fascinating field for speculation. It is possible, of course, but improbable, that a

[1] Delivered at the Marine Biological Laboratory, Woods Hole on August 19, 1930.

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THE SECOND REYNOLD A. SPAETH MEMORIAL LECTURE[1]

Protein Synthesis

HARDOLPH WASTENEYS

Department of Biochemistry, University of Toronto, Toronto, Canada

It is not necessary to elaborate on the importance of an understanding of the mechanisms involved in the enzymatic synthesis of proteins, because it is perhaps the most fundamental characteristic of living matter, just as protein itself is the most characteristic constituent of protoplasm.

We know something of the intermediate and a good deal of the final products of degradation of the protein molecule, though we know little or nothing of the exact structure of the molecule itself.

In the higher organisms which, unlike the plants, have not the power to synthesize amino acids, material for the synthesis of the proteins required for growth and maintenance must be ingested in the form of proteins or amino acids. The proteins, before final absorption, must be broken down in the digestive tract into the constituent amino acids by means of enzymes, and these amino acids are built up in the body into the infinite variety of proteins characteristic of the various organs and tissues.

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specific enzyme performs each synthesis. Experiments which I shall describe this evening provide at least a possible alternative.

The first part of the problem to be attacked was, of course, the synthesis of protein from its degradation products by means of enzymes. It was then necessary to prove that a real synthesis had been achieved. The investigation and control of the conditions concerned in the synthesis was next attempted, and finally the possibility was investigated of producing from one substrate, with a single enzyme, proteins of different composition.

Those of our own experiments to which I shall refer were performed in collaboration chiefly with Dr. H. Borsook, now of the California Institute of Technology, and I shall always look back with pleasure to the many hours which we spent together in these investigations.

I should explain, perhaps, at the outset that we were unable to obtain synthesis from anything less complicated than peptic digests of various proteins, although it is possible to obtain synthesis from such substrates with several enzymes. The experiments do not, therefore, reproduce an *actual biological* synthetic process. Investigations into the mechanism of peptic synthesis and its control are, however, at least very suggestive as to the probable mechanism of the biological synthesis.

First let me say a few words about the theoretical aspects of enzymic synthesis. In applying the law of mass action to hydrolysis in general, it is usual to write:

$$A + H_2O \rightleftharpoons \text{products}$$
$$e.g.\ \text{ester} + H_2O \rightleftharpoons \text{alcohol} + \text{acid}$$

and the equilibrium equation in this case becomes

$$\frac{(\text{alcohol})\ (\text{acid})}{(\text{ester})\ (\text{water})} = \frac{K_1}{K_2} = K.$$

In such a system it is necessary, in order to obtain synthesis, to diminish the concentration of water as far as possible, and it has been assumed that the synthetic processes taking place in living cells include a very efficient mechanism or mechanisms for accomplishing this, such as surface condensation or imbibition by colloids. In our first experiments with protein synthesis *in vitro* the attempt was made to produce such surface condensation by means of the artificial cells used by T. B. Robertson and E. Newton Harvey. Robertson, you remember, used them to illustrate the mechanics of cell division and Harvey to demonstrate permeability. We were not successful in obtaining synthesis by this means alone but we returned to a similar procedure in later experiments, with interesting results which I will try to describe towards the end of this lecture.

To return to the question of concentration of water. In the alcohol-acid-ester system the molecular concentrations of water and of the other reacting components are of somewhat the same order of magnitude, but in protein systems, at least *in vitro*, the molecular concentration of water, even in very concentrated solutions, must be enormously greater than that of the other reacting components; and if we had to consider concentrations in the same terms as for the alcohol-acid-ester system, it would be impossible to attain syntheses *in vitro* because the relatively large molecular concentration of water would always send the reaction in the direction of hydrolysis. This is one reason why, under ordinary conditions of protein hydrolysis, the reaction is always complete, *i.e.*, the equilibrium position appears to be, unlike the ester system, at 100 per cent hydrolysis.

But in the hydrolysis of proteins and also, to a lesser extent, of fats, we have another factor, which alters the picture and minimizes the relative importance of the molecular concentration of water. This factor is the formation from one molecule of substrate (protein or fat), of a number of molecules of hydrolysate (amino acids or glycerine and fatty acids, as the case may be).

Hydrolyses of this type are in a special category, because the formation of *many* molecules from *one* molecule leads to other consequences, which were, as far as I know, first pointed out by Benjamin Moore. I will very briefly review his suggestions.

Moore deduced on thermodynamical grounds that the conditions of equilibrium for the reaction $A \rightleftarrows B$ may be expressed by the equation

$$P_2 = KP_1 ,$$

where P_2 and P_1 are terms proportional to the osmotic pressures of A and B respectively.

When, however, one molecule decomposes into two, *i.e.*, $A \rightleftarrows 2B$, as in the hydrolysis of an ester, the equilibrium reaction may be expressed by the equation

$$P_2^2 = KP_1 .$$

No term is inserted for the water which takes part in the reaction because its molar concentration remains practically constant.

For such simple reactions as $A \rightleftarrows B$ the ratio of the concentrations at equilibrium of A and B is a constant, and is independent of the absolute concentration, as the expression $P_2 = KP_1$ indicates. Consequently dilution or concentration of the system does not alter the proportions of the components at equilibrium, but where one molecule decomposes into *more than one molecule*, the equilibrium ratio is not constant and is not independent of the absolute concentrations. For example, if in the equilibrium reaction $P_2^2 = KP_1$, the concentration is doubled,

$$\text{then } P_1 \text{ becomes } 2P_1$$
$$\text{and } P_2^2 \text{ becomes } 2P_2^2,$$

but since P_2 is the $\sqrt[2]{\ }$ of the left-hand term,

$$P_2 \text{ becomes } \sqrt{2P_2^2} \text{ or } \sqrt{2} \times P_2$$

i.e., while P_1 has been doubled in concentration, P_2 has been only increased $\sqrt{2}$ or 1.4 times, so that under these conditions, when the concentration of substrate is doubled, the concentration of hydrolysate is less than doubled at equilibrium. In other words, the degree of hydrolysis is less, or in still other words, the tendency towards synthesis is greater. Conversely, on dilution, P_2 decreases in less ratio than P_1, that is, the degree of hydrolysis increases. This effect, in the case of substances like esters and disaccharides, is not very marked; it is more marked in the case of simple fats where, since the fat molecule decomposes into four molecules, the equilibrium reaction becomes

$$P_2^4 = KP_1,$$

and it is of course very marked in the case of proteins, where the value of the exponent is much higher.

The consequences of this relationship can be briefly stated as follows: In reactions in which one molecule is decomposed into a number of molecules the position of the equilibrium point depends on the absolute concentration of the solution. In concentrated solutions of such systems these reactions are incomplete,—there is a tendency towards reversion or synthesis. In dilute solutions these reactions tend to be complete and irreversible, *i.e.*, the tendency towards hydrolysis is great.

These effects of concentration and dilution apply with especial force in the case of proteins and of polysaccharides. They are independent of the effect of concentration on the molecular concentration of the water itself (which, as we have seen, cannot be practically reduced for proteins *in vitro* to proportions which would drive the reaction in the direction of synthesis), and they have two consequences: First they enable us to easily attain complete hydrolysis of proteins in dilute solutions, and second, they enable us to attain synthesis with great ease in concentrated solutions, eliminating the necessity for mechanisms for reducing the molecular concentration of water to dimensions commensurate with the molecular concentration of other components. Since presumably the same considerations apply *in vivo,* we no longer need to postulate special biological mechanisms for effecting syntheses.

It can also be deduced on thermodynamical grounds, as Moore pointed out, that an increase in temperature should favour synthesis and a decrease should favour hydrolysis. The method for the synthesis of protein in a peptic digest of protein is then very simple: It consists simply in concentrating the digest, raising the temperature and adding pepsin. The $[H^+]$ must also be controlled. Synthesis begins immediately and equilibrium is attained in a few hours. Under optimal conditions a yield of 50 per cent of protein has been obtained.

Since 1886, when the first synthesis of protein-like material was carried out by Danilewski, many workers have obtained synthesized protein. Sawjalow in 1901 gave to the protein precipitate the name plastein, and most of these protein precipitates have since been known by that name.

The first unequivocal proof that the precipitate obtained is a synthetic product was furnished in 1911 by Henriques and Gjaldbäk, who demonstrated that plastein formation is accompanied by a decrease in free animo-nitrogen, the converse of the common method of following protein hydrolysis by estimating the increase in free amino-nitrogen. We confirmed these results and it can be shown that the free COOH groups also decrease in a digest on plastein formation, and, as Rona has shown, exactly parallel the reduction in free amino groups.

The plasteins may be hydrolyzed by pepsin in the same manner as native proteins, yielding proteoses and peptones. Plastein, like native protein, is precipitated from its solution by trichloroacetic acid, which will not precipitate gelatin nor the proteoses, so that its complexity must be of the order of *native* protein. It gives, of course, all the usual protein reactions. Perhaps the best proof of synthesis is obtained from a fractional analysis which we made of digests where different amounts of plastein had been synthesized. We found that all fractions of the digest contribute to the formation of plastein.

There is no doubt then that these plasteins represent synthesized protein-like material. Their identity has been doubted by many because they apparently differ in physical properties (more especially in their insolubility) from the native proteins or, particularly, the proteins from which the digest is prepared. As a matter of fact their insolubility appears to be due to a denaturation which results from the conditions in which they are formed. Albumin and other soluble proteins under similar conditions, that is, in the presence of a concentrated digest at the optimum pH of 4.0, are rendered as insoluble as plastein.

We have then the first part of the simple problem completed. We

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have synthesized protein and we have satisfied ourselves that the product is indeed protein and that it is synthesized from all the principal components, *i.e.*, that the proteose, peptone and sub-peptone constituents all contribute to the rebuilding of a protein or protein-like substance.

The next part of the problem is the investigation of the conditions under which the synthesis is obtained. This has yielded results of the greatest interest to us though I can discuss them only very briefly in this lecture.

In studying the effects of concentration on synthesis, we found the following relations. The limiting concentration below which no synthesis could be made to occur corresponds to the equivalent of about eight per cent of protein. Synthesis takes place at all concentrations greater than 8 per cent. With concentrations higher than 8 per cent there is a straight line relationship between the amount of protein synthesized and the concentration of material in solution: synthesis is in simple inverse proportion to the dilution. At high concentrations, however, that is, at concentrations which correspond to more than 40 per cent of protein, there is a rapid falling off, until at still higher concentrations no synthesis is obtained. We have no explanation for this falling off, but a similar phenomenon was observed by Armstrong and Gosney in the enzymatic synthesis of fats.

These results on the effect of concentration confirm Moore's prediction that the important factor in the enzymatic synthesis of proteins is the concentration of material in solution, and not the relative concentration of water. It can also be shown that as he predicted, the same holds true for hydrolysis: the tendency for hydrolysis diminishes as the concentration of protein increases, and in concentrated solutions of proteins, hydrolysis is incomplete no matter how much enzyme is used.

A study of the effect of the addition of proteins in each case shows that we are dealing with true equilibria in these reactions. Our experiments demonstrate that if previously synthesized protein is added to the system, synthesis is inhibited to an extent directly proportional to the amount of added protein, which also shows that the plastein takes part in the equilibrium as if it were in solution. Conversely it can be shown that addition of products to a digest in which hydrolysis of protein is going on inhibits hydrolysis to an extent proportional to the amount of products added.

I mentioned earlier that Moore had deduced on thermodynamical grounds that in reactions of the type of protein synthesis and hydrolysis, the effect of temperature on the equilibrium position is such that increase of temperature favours reversibility, *i.e.*, increase in temperature favours synthesis and decrease favours hydrolysis.

We were able to confirm this deduction by a very striking experiment. A solution in which hydrolysis was proceeding at a temperature between 20°–24° was adjusted to the optimum pH for synthesis. It was then divided into three portions. One was maintained at 20° and showed the further change in the direction of hydrolysis to be expected under the circumstances. The second portion was raised to 65° C. In ten minutes plastein began to form and in 30 minutes considerable protein had been synthesized. A third portion was boiled to destroy the enzyme and then maintained at 65° C. for 8 hours without the occurrence of any change except the faint coagulation produced on boiling.

In this experiment then we have a solution in which hydrolysis is proceeding at 20° C., and simply by raising the temperature we are able to induce synthesis. We find that the equilibrium amount of protein synthesized increases with increasing temperature up to 72° C., at which temperature the enzyme is destroyed; that is, the effect of increase of temperature is to move the equilibrium point more and more over to the protein side.

So far I have made hardly any reference to the effect of hydrogen ion concentration on peptic synthesis. As you could guess, it is a most important factor. Practically all the previous work on protein synthesis was performed before the importance of the $[H^+]$ in enzyme reactions had been realized, and before methods for its exact determination had been developed. The only determination, that of Henriques and Gjaldbäk, had placed the optimum for synthesis with the optimum for hydrolysis at a pH of 1.5. On many occasions we determined the optimum for synthesis to be at a much more alkaline reaction, *viz.*, pH 4.0. It has been shown by Oda that this optimum holds, regardless of the acid employed, and that it is unaffected by the presence of a great variety of salts.

We have tried to determine the mechanism by which the $[H^+]$ controls the extent of peptic synthesis. The following interesting fact was discovered: If we construct a curve, plotting the yields obtained at different $[H^+]$, and plotting the yields obtained as per cent with the yield at the optimum pH as 100 per cent, the curve resembles in form and in slope either the primary dissociation curve of a dibasic acid or the undissociated residue of an amphoteric electrolyte. It seems probable from this resemblance, or correspondence,—because the correspondence is remarkably exact,—that the amount of protein synthesized at any given $[H^+]$ is dependent on the degree of ionization of some component of the system. It can hardly be the ionization of the enzyme which is concerned, because, as Northrop has shown, pepsin exists as a monovalent anion between pH 1–7, and changes in pH within that range do not affect the degree of dissociation. It might be an effect on the autodestruction of the enzyme, but at least within the range of pH 1–4 there is no autodestruction under the experimental conditions. One can only suppose that the result is to be interpreted as showing the relation between synthesis and ionization of the digest itself; but titration curves of the digest show that the ionization of the digest as a whole is not represented by these curves, and one is forced to the conclusion that since there is this remarkable correspondence between the experimental values and a theoretical dissociation curve, and since we have ruled out other possibilities, the protein synthesized at any given $[H^+]$ is dependent on the degree of ionization of one component of the digest or rather, of the substrate.

Another respect in which peptic synthesis behaves rather differently from other enzyme reactions is in the effect of varying enzyme concentration on the equilibrium position. It has been found by other observers, and confirmed by the writer, that the equilibrium amounts of protein synthesized are increased with increasing amounts of pepsin. This is, of course, an exception to the general law of enzyme action and catalysis, which always assumes that the equilibrium position is not affected by the concentration of the enzyme. The explanation of this effect of varying concentrations of enzyme is suggested by another series of experiments which we carried out on the effect of the presence of emulsions in the system on peptic synthesis. These are the experiments I referred to earlier as resembling the experiments where the substrate was concentrated at the surface of artificial cells. We found that certain emulsions, such as those formed when chloroform, benzene, benzaldehyde, etc., are shaken up with the digest, accelerated the attainment of equilibrium. Moreover, in the presence of some emulsions, such as benzene, toluene, benzoic acid and benzaldehyde, synthesis is effected even in the absence of enzyme.

Now we may consider that pepsin, being a colloid or in colloidal association, owes its action, in part at least, to properties resembling those possessed by these emulsifying agents. This action occurs by virtue of the presence of the interphase surface. The action of emulsifying agents further resembles that of pepsin in that increasing amounts, under certain conditions, also augment the yield of protein synthesized at equilibrium. This effect of concentration of enzyme or emulsifying agent on the equilibrium is consistent with thermodynamical conceptions, since the addition of an emulsifying agent means an increase in the interphase surface, and this increase in surface means in turn an increase in surface energy to the system because of the increased amount of surface energy available. Thus, increasing the concentration of pepsin should have the same effect as that obtained when energy in any other form is added to the system, as, for example, by increasing temperature. We have already seen that increasing the temperature up to the limit of destruction of the enzyme has a similar effect. On this basis we can therefore explain the departure from the classical conception that the concentration of enzyme should have no effect on the amount synthesized at equilibrium.

There is another point of similarity in the action of pepsin and of emulsions. T. B. Robertson, in 1926, discussed the possibility that concentration of the reactive groups of amino acids by orientation at lipoid surfaces might account for the completeness of enzymatic synthesis in living tissues. As we have just seen, in certain cases synthesis can be effected even without enzyme by the emulsifying agent alone if it is an accelerator of synthesis. Some of these emulsifying agents, *e.g.*, benzene and toluol, are chemically inert compounds. They can hardly enter into chemical combinations with digest constituents, and they are readily separable again from the synthesized protein. Their effect is almost certainly due to a specific orientation of the components of the digest which participate in the synthesis. That this action is specific is shown by the fact that the chemical and physical properties of the proteins synthesized vary with the means employed in synthesis. The base-combining properties, the ratios of free amino and free carboxyl groups to total nitrogen, the hydrolysability by pepsin, all show marked differences which are reproducible.

The solubilities also show very marked differences, *e.g.*, proteins synthesized by pepsin with or without benzaldehyde are soluble only at alkalinities greater than pH 7.7; at 7.6 the proteins are precipitated almost completely.

Proteins synthesized with benzaldehyde in the absence of pepsin are not precipitated until the reaction reaches pH 6.0, and the benzene-synthesized protein is not precipitated until the pH reaches 5.0. At more acid reactions these proteins are also completely precipitated.

These results indicate that, presumably through variations in the orientation of the participating components of the digest, proteins of *different chemical composition* are synthesized. They suggest also a possible explanation for the specificity of the action of the enzymes themselves.

We have been able to work out other interesting suggestions from studies of the conditions of peptic synthesis, but time will not permit me to discuss these results.

It is unfortunate that we have had very little success with protein synthesis from digests less complex than peptic digests, but we still hope to obtain synthesis from simpler substrates, and are continuing our studies.

Peptic synthesis, therefore, is at best only a possible model of the biological synthesis of proteins. However, it provides many interesting suggestions as to the mechanism of the biological process, and not the least important of these is the last discussed, namely, the suggestion that specific orientation on the colloidal interphases provided by the proteolytic enzymes and their associated colloids may best account for the ability of the organism to synthesize its many varieties of proteins from a common substrate.

STUDIES ON AMPHIBIAN METAMORPHOSIS

IX. INTEGUMENTARY SPECIFICITY AND DERMAL PLICÆ FORMATION IN THE ANURAN, RANA PIPIENS

O. M. HELFF

(From the Department of Zoölogy, State University of Iowa, and the Department of Biology, University College, New York University)

INTRODUCTION

The dermal plicæ or folds of newly-metamorphosed *Rana pipiens,* as is well known, constitute a striking and characteristic development for this species. Although minor folds have been described, the most distinct plicæ are those of the dorso-lateral trunk and lateral upper jaw regions. The former consists of two folds, one on each side of the trunk, running from the dorsal-posterior border of the eye back to a point just lateral to the urostyle prominence. The latter likewise consists of two folds, one on each side of the upper jaw, running from a point just anterior and ventral to the external nares posterior on a level just ventral to the tympanic membrane to a point just dorsal to the origin of the fore-limb. The folds are usually about 0.5 mm. in width at this time and are white in color in sharp contrast to the black or dark-greenish coloration of the adjacent integument. Histologically, the folds are seen to be composed of integument having a much thickened stratum spongiosum in which are found mucous and unusually large poison glands. It is the concentration and abnormal size of these glands in this particular region which result in the formation of an actual ridge or fold as viewed externally.

The development of the dermal plicæ during metamorphosis may be roughly estimated by external observation. In the normal larva, just prior to the onset of involution, no indications of plicæ formation are visible. During the early stages of transformation, however, broad though very indistinct bands of integument are noticeable in the dermal plicæ regions. These are at first of a dull, grayish-white shade. Later, however, towards the close of metamorphosis, they become lighter in color, as the bands appear to narrow, and more sharply demarcated from the adjacent integument. Following larval transformation, the plicæ become increasingly whiter and very distinct, after which they undergo relatively little change during the early growth period of the young frog.

The embryology of the mucous and poison glands as they occur in amphibian integument has interested many workers and given rise to numerous conflicting results and interpretations. Practically all workers, however, agree that the glands have an ectodermal origin, being derived from the Malpighian layer of the epidermis. The point of difference seems to lie chiefly in whether or not the two types of glands have a common origin or arise from separate sources. Separate developments have been described by Schultz (1889) and Nirenstein (1908) in *Salamandra,* by Heidenhain (1893) and Nicoglu (1893) in *Triton,* by Engleman (1872) and Seeck (1891) in the frog, and by Dawson (1920) in *Necturus.* Common origins, which in some cases have regarded the mucous and poison glands as simply different developmental stages of one and the same gland, have been described by Ancel (1902) in *Salamandra,* by Fano (1903) in *Triton,* by Esterly (1904) in *Plethodon,* by Wenig (1913) and Calmels (1883) in the toad, and by Szczesny (1867), Junius (1896), and Weiss (1908 and 1915) in frogs.

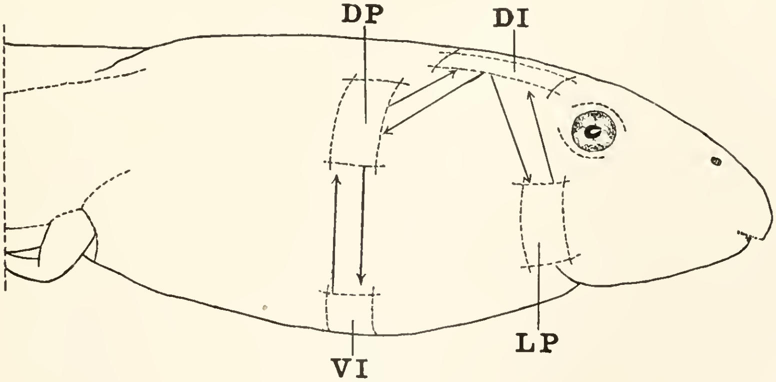
Although the present work has indicated certain points regarding the embryology of the acinous glands as they occur in *Rana pipiens,* the main object in view was to determine to just what extent various integuments of the larva are specific for glandular formation as found in dermal plicæ regions. Histologically, all integument of the larva, prior to the onset of metamorphosis, is similar in that there is an absence of acinous glands or of any indication of their future development. The question naturally arises, therefore, whether or not the integument of the dermal plicæ regions is hereditarily specific for the development of the glandular picture as it is found during larval involution. Conversely, the possibility suggested itself that all integument might be so stimulated to unusual glandular development, if located in the specific regions characterized by dermal plicæ formation during metamorphosis. The above points were subjected to analysis by making suitable integumentary transplantations. In general, these consisted of the autoplastic transplantation of larval integument from the dorso-lateral and lateral dermal plicæ regions to the back or belly, and vice versa. The development or non-development of dermal plicæ tissue in such transplants during larval involution could then be studied and information relating to integumentary specificity in this respect obtained.

The writer wishes to express his appreciation for the facilities tendered him during the summer of 1929 by the Iowa Lakeside Laboratory, Milford, Iowa, where the operations described in the present paper were made.

MATERIALS AND METHODS

The stock used for all operations consisted of large *Rana pipiens* tadpoles collected in the vicinity of Spirit Lake, Iowa, during the months of June and July, 1929. Prior to the onset of metamorphosis the larvæ of this particular strain of *Rana pipiens* grow to the unusual size of 110–115 mm. total length. When collected and operated on, however, they averaged about 80 mm. in length, with hind limbs 8 to 12 mm. long. The selection of larvæ in a stage at least three weeks prior to the earliest signs of metamorphosis was essential, since preliminary sectioning of integument had shown that the acinous glands begin their development at about the beginning of the metamorphic period.

The various integumentary transplantations were made as follows: The larva was first anæsthetized in a 0.05 per cent aqueous solution of chloretone. The approximate regions of the integument in which dermal plicæ development later occurs were next located and a rectangular piece of skin, large enough to include a small amount of the adjacent integument above and below the dermal plicæ region, was cut and removed. The fact that all integument, including that of the dermal plicæ regions, is of the same macroscopic appearance, necessitates a knowledge of the approximate level of integument which will later develop into dermal plicæ, in order that the skin graft will include all of the potential plicæ integument. A similar sized piece of integument was now removed from either the mid-dorsal or mid-ventral region of the same larva and transplanted to the wound area left subsequent to the removal of the dermal plicæ skin graft. The latter was now transplanted to the area exposed by the removal of the back or belly integument (text fig. *A*). Following this, the larva was placed in shallow water so as to expose the two grafted areas to the air, thereby hastening



TEXT FIG. A

the adhesion of the transplants. Following adhesion of the transplants, the larva was immersed in an individual aquarium and allowed to recover from the effects of the anæsthetic. Subsequent treatment included feeding with *Spirogyra* to induce growth and daily observations to record the onset of metamorphosis and the development of macroscopic signs of dermal plicæ formation in the various transplants during larval involution. Representative skin transplants were removed at various stages of metamorphosis in order to determine the development or non-development of glandular structures.

RESULTS

Normal Histological Development of Dermal Plicæ Structures

Although the normal development of the acinous glands has been pictured and described by many authors in a variety of amphibians, relatively few have dealt with the formation of these glands as they occur in the dermal plicæ and none, with the possible exception of Massie (1894), has dealt with the conditions as found in *Rana pipiens*. It is assumed that Massie probably worked with *Rana pipiens*, although the species of frog tadpole used was not specifically stated. The exact mode of the various structural differentiations in relation to the results of skin transplantation as described in the present paper was not so important to determine as was a knowledge of the histological picture of the dermal plicæ typical of various stages of larval involution.

For convenience, three stages of glandular development in dermal plicæ regions corresponding with three stages of larval involution will be briefly described. These descriptions apply especially to the dorsolateral dermal plicæ.

Stage 1. *Larvæ within One Week of Onset of Metamorphosis.*—The larvæ are in all respects typical tadpoles. There are no macroscopic signs of dermal plicæ formation. Histologically, all layers of the integument appear normal as regards thickness and pigmentation. A few, widely-scattered, oval-shaped, embryonic gland " nests " are to be found partly embedded in the lower layers of the epidermis and invaginated into the stratum spongiosum. The " nests " appear to be composed of large, oval-shaped nuclei and cells having large nuclei with a minimum of cytoplasm. Mitotic figures are present in some of the cells. The " nests " all appear to resemble one another cytologically. It is clear that the component cells have been derived from the Malpighian layer of the epidermis, probably for the most part from the nuclei.

Stage 2. *Larvæ in Early Stage of Metamorphosis.*—The visible external metamorphic changes of the larva include growth of the hindlimbs and slight atrophy of the tail. Otherwise, the larva is still tadpole-like in most respects. Externally, the dermal plicæ are indicated by broad, indistinct bands of a grayish coloration. Histologically, the epidermis is unchanged in thickness but appears to be less pigmented. The stratum spongiosum is considerably thickened to accommodate the glands. The poison glands are fairly well developed and of a good size. The muscular and fibrous layers of the latter are present. Practically all have ducts developed. The inner epithelial layer is composed of cuboidal-shaped cells, while large granules occupy the central cavity of the gland. The glands are definitely poison glands. Small mucous glands are also found, some of which have acquired ducts. In addition, glandular " nests " are also found in the usual location, the inference being that they are developing mucous glands. The adjacent back integument now possesses a few, widely scattered gland " nests," which appear smaller than those described in Stage 1 dermal plicæ.

Stage 3. *Larvæ near End of Metamorphosis.*—The animals are typically frog-like, although the tympanic membranes are not as yet fully developed. The tail has atrophied to a small stump. The dermal plicæ appear narrower, distinctly marked off from the adjacent integument and grayish-white in color. Histologically, the epidermis has thickened and contains more layers than that of the adjacent back integument. The stratum spongiosum is especially thickened, while the melanophores in this layer and the epidermis appear to be considerably less evident. The integument as seen in cross-section is markedly convex along its epidermal surface. The glands are much larger than hitherto described. The poison glands show considerable variation in size, while the epithelial cells lining the base of the gland cavity are usually columnar in shape. The mucous glands are more numerous and larger than in Stage 2, while glandular " nests " may still be found, especially towards the edges of the dermal plicæ.

Further developments, following Stage 3, include the general whitening of the plicæ as viewed externally, while histologically the glands (especially the poison glands) increase still further in size and tend to crowd together to form the arrangement typical of the fully developed dermal plicæ.

Reciprocal Transplantation of Dermal Plicæ Integument with that of the Back and Belly

Series A.—This series consisted of reciprocal, dorso-lateral dermal plicæ and back skin transplantations (*DP* and *DI*, text fig. *A*). Fifty-

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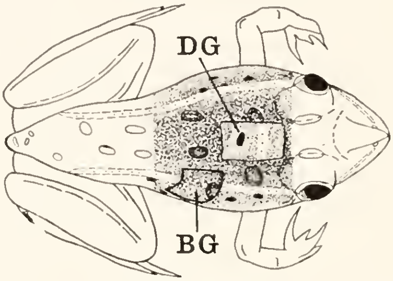
seven larvæ were operated on in all. During metamorphosis, it was soon evident that typical macroscopic signs of dermal plicæ formation were present in the grafts transplanted to the back. If the graft had been transplanted in the same orientation as it had previously occupied in relation to the anterio-posterior axis of the body, the dermal plicæ developed, parallelling the normal ones of the animal. If, however, the graft had been rotated 90° during transplantation, then the dermal plicæ developed at right angles to the normal ones of the animal. The narrowing and whitening of the dermal plicæ band of integument progressed at a uniform rate identical with the normal formation of the dorso-lateral dermal plicæ on the same animal (*DG*, text fig. *B*). It was also observed that the general pigmentary changes occurring in the transplant were characteristic of the region of integument from which the graft had been secured. The back skin grafts transplanted to dermal plicæ regions underwent macroscopic changes in coloration, including the development of spots, and corresponding in all details to the pigmentary changes of the integument of the back. There were no external indications of dermal plicæ formation, not even where the edges of the grafts were in contact with the developing normal dorso-lateral dermal plicæ (*BG*, text fig. *B*). Following the complete resorption of the tail, the metamorphosed animals generally lived for about three weeks, at which time they died due, no doubt, to lack of proper food. Representative skin transplants were removed at this time and sectioned for histological study.



Text Fig. B

The microscopic appearance of dermal plicæ integumentary grafts transplanted to the back is represented in Fig. 1, Plate 1. It will be noted that typical glandular development and tissue thickening has occurred, corresponding in all details to the condition typical of normal dorso-lateral dermal plicæ at this stage of the animal's life. The various histological features are representative of a somewhat later stage, as previously described for Stage 3 of dermal plicæ development. The epidermal and stratum spongiosum layers are especially thickened, giving the external surface of the dermal plicæ integument a decidedly convex shape. The poison glands are larger than those described for Stage 3 and more closely crowded together. Usually five or six are found across the width of the plicæ. The mucous glands are relatively much smaller and fewer in number and occupy only the upper regions of the stratum spongiosum. The microscopic appearance of the back skin grafts transplanted to dorso-lateral dermal plicæ regions is represented in Fig. 2, Plate 1. Again the various histological structures correspond to the conditions typical of normal back integument at this time. The epidermis is normal in thickness, while the stratum spongiosum is but moderately thickened to accommodate the glandular development. Regarding the latter, the mucous glands appear to predominate both in size and number. In fact, the mucous glands are larger and more numerous at this time than is true of normal dorso-lateral dermal plicæ. Only a few, small, widely-scattered poison glands are present, which have probably just begun their development (*PG*, Fig. 2, Plate 1). It should be emphasized, however, that this same histological picture is typical of normal back integument at this time.

Series B.—Series B consisted of reciprocal, dorso-lateral dermal plicæ and belly skin transplantations (*DP* and *VI*, text fig. *A*). Ten larvæ were operated on in all. The dorso-lateral dermal plicæ integumentary grafts developed normal dermal plicæ during metamorphosis which, together with the characteristic pigmentation pattern of the adjacent transplanted back skin, presented a striking contrast to the surrounding white belly skin (Fig. 5, Plate 1). Histologically, the same glandular and integumentary conditions were found as already described for this type of graft when transplanted to the back (see Series A, above). The belly skin transplants were typically pure white in color when transplanted to the dorso-lateral dermal plicæ regions. This lack of melanophore pigmentation and spotting was maintained during subsequent larval involution and likewise presented a strong contrast as compared with the darkly pigmented adjacent skin of the back and side (Fig. 6, Plate 1). The histological appearance of such grafts, at a time when the normal dermal plicæ were well formed, is represented in Fig. 3, Plate 1. Compared with back skin grafts transplanted to this region, the belly skin transplants were somewhat thinner as a whole, while the mucous glands were correspondingly smaller though more numerous. The poison glands were very small and scarce. In general, however, it may be said that the microscopic appearance was identical with the normal integument of the belly at this time.

Series C.—This series consisted of reciprocal, lateral (upper jaw) dermal plicæ and back skin transplantations (*LP* and *DI*, text fig. *A*).

In all, twenty larvæ were operated on. The development of typical macroscopic signs of dermal plicæ formation were found to occur during involution in the transplants made to the back. The appearance was quite similar to the dorso-lateral transplants to the back as illustrated in text fig. *B*, except that the whitening of the integument was slightly delayed and the width of the plicæ bands somewhat narrower. This, however, is typical of normal lateral plicæ development. The back skin grafts transplanted to lateral dermal plicæ regions, like the back skin transplants of Series A, failed to exhibit visible macroscopic signs of plicæ formation during larval involution. Histologically, the appearance of these transplants was also similar to that described for the back skin transplants of Series A and pictured in Fig. 2, Plate 1. The microscopic appearance of the lateral dermal plicæ transplants on the back presented several differences as compared with the dorso-lateral dermal plicæ transplants described in Series A and pictured in Fig. 1, Plate 1. Figure 4, Plate 1, represents a typical section taken through one of these transplants at the usual time,—some three weeks following the completion of larval involution. Perhaps the greatest difference observed was in the relatively greater abundance and size of the mucous glands. The poison glands, on the other hand, were smaller and less numerous than in the dorso-lateral dermal plicæ transplants, being evidently less developed. The fact that they were usually larger than the mucous glands,

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FIGS. 1 TO 6. *BI*, adjacent integument of back; *CE*, columnar epithelial cells; *DG*, dorso-lateral dermal plicæ skin graft transplanted to belly; *DI*, integument of dorso-lateral trunk region; *DP*, normal dorso-lateral dermal plicæ; *DPG*, dorso-lateral dermal plicæ developed in skin transplanted to the belly; *E*, thickened epidermis of dermal plicæ; *G*, granules of poison gland; *MG*, mucous gland; *P*, pigment masses; *PG*, poison gland; *T*, thickened stratum spongiosum of dermal plicæ; *VG*, belly skin graft transplanted to dorso-lateral dermal plicæ region; *VI*, integument of the belly.

FIG. 1. Histological section through dermal plicæ developed in skin graft from dorso-lateral dermal plicæ region previously transplanted to the back. Transplant sectioned three weeks following close of larval metamorphosis. × 112.

FIG. 2. Histological section through back skin graft previously transplanted to dorso-lateral dermal plicæ region. Transplant sectioned three weeks following close of larval metamorphosis. × 224.

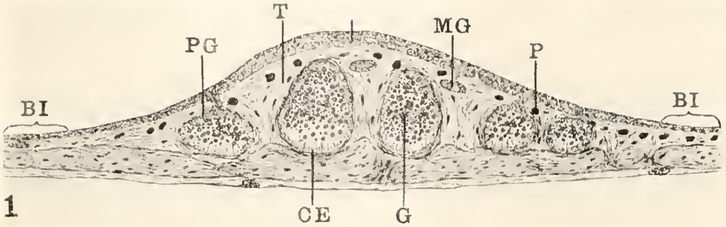
FIG. 3. Histological section through belly skin graft previously transplanted to dorso-lateral dermal plicæ region. Transplant sectioned three weeks following close of larval metamorphosis. × 336.

FIG. 4. Histological section through dermal plicæ developed in skin graft from lateral (jaw) dermal plicæ region previously transplanted to the back. Transplant sectioned three weeks following close of larval metamorphosis. × 112.

FIG. 5. Macroscopic appearance of skin graft from dorso-lateral dermal plicæ region, previously transplanted to the belly, three weeks following the close of larval metamorphosis. × 10.

FIG. 6. Macroscopic appearance of belly skin graft, previously transplanted to dorso-lateral dermal plicæ region, three weeks following the close of larval metamorphosis. × 10.

PLATE I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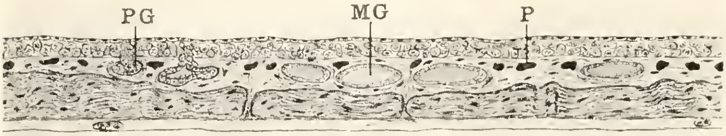

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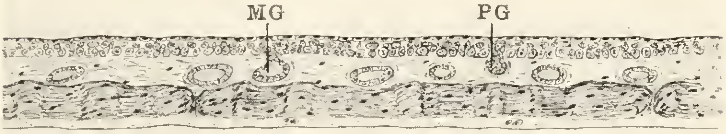

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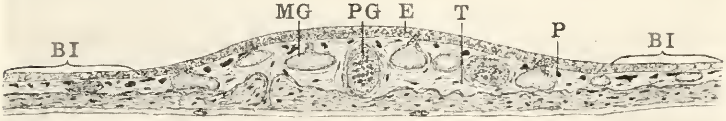
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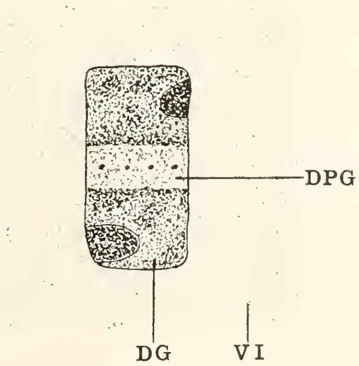

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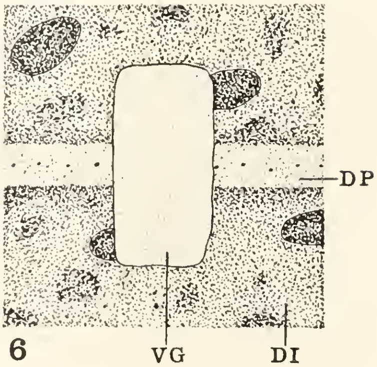

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however, probably accounted for the thickening of the stratum spongiosum present. The slight thickening of the epidermis and the moderate increase in thickness of the stratum spongiosum resulted in a convexity of the epidermal surface of the integument which, however, was not nearly as pronounced as in the case of the dorso-lateral dermal plicæ transplants. In general, it can be said that the histological appearance of the lateral dermal plicæ transplants on the back corresponded very closely to the microscopic picture of the normal lateral dermal plicæ of the same animal at this time.

Discussion

The results of the present work confirm those of Massie (1894) for the frog tadpole and of Wilder (1925) for *Eurycea* larvæ in that no anlagen of the acinous glands appear until just prior to the onset of metamorphosis. Likewise, the evidence is strongly indicative that they are derived from the Malpighian layer of the epidermis, separate developments being necessary for the two types of glands. There is no indication in the histological sections that transformation ever occurs from mucous to poison gland. In fact, as regards the dorso-lateral dermal plicæ regions especially, there is every reason to believe that the poison glands develop first and contain typical granular inclusions in the lumen at a time when the mucous glands are still in the anlage stage.

Study of the dermal plicæ in various stages of development leads to the conclusion that the progressive whitening of dermal plicæ integument is due to several factors. Of these the gradual reduction in the number of melanophores both in the epidermis and stratum spongiosum is probably of primary importance. This reduction in pigmentation apparently more than counterbalances the effects of increased thickening of the epidermis and stratum spongiosum. The latter layer, it may be stated, is apparently fully as dense in dermal plicæ regions as it is elsewhere throughout the integument. The extraordinary large size of the poison glands in dermal plicæ regions may also help to give the integument a certain degree of transparency and so facilitate the development of the characteristic white coloration of the plicæ as viewed externally.

The results of reciprocal, dermal plicæ skin transplantations with integument of the back and belly are especially clear in that certain narrow bands of larval integument are evidently physiologically specific for the development of mucous and large poison glands during larval involution. This specificity would also seem to include the ability to reduce pigmentation and to thicken the epidermis and stratum spongiosum. Apparently, therefore, neither the adjacent musculature or other local factors have any influence on the integument in the development of dermal plicæ structures. This is borne out by the fact that back and belly skin transplanted to dermal plicæ regions failed to develop the slightest tendency towards dermal plicæ characteristics and that integument from dermal plicæ regions was capable of forming typical dermal plicæ structures at the usual metamorphic stage when transplanted to foreign locations. Logically, this specificity must be determined at some stage in the development of larval integument, the probability being that it is completed at a relatively early growth period of the larva, possibly at the same time that the integument of the embryo is formed. It would be interesting, in this connection, to make suitable integumentary transplantations on newly hatched tadpoles.

Granting that certain bands of integument are definitely specific for dermal plicæ formation during larval involution, the question naturally arises regarding the possible causative influences which evoke this differentiation and growth. It would seem probable that these influences are hormonic in nature and hence transported through the blood stream when released at a definite stage of metamorphosis. Possibly only an initial hormonic stimulus is necessary to bring about development of dermal plicæ structure or it may be that continued stimulation of this nature is essential for complete differentiation. Suitable homoplastic transplantations of integument, including half-formed dermal plicæ, to non-metamorphosing larvæ would undoubtedly answer this question.

The apparently direct influence of metamorphic hormones through the blood stream has been shown to effect the differentiation and growth or degeneration of other larval structures during anuran metamorphosis. The transformations so effected include the differentiation and growth of the fore-limbs (Helff, 1926), the hind-limbs (Schubert, 1926), the tongue (Helff, 1929*a*), the columella (Helff, 1929*b*), the nictitating membrane (Lindeman, 1929*a*) ; the histolysis of tail muscle (Helff and Clausen, 1929, and Clausen, 1929), and of tail integument (Lindeman, 1929*b*, and Clausen, 1929) ; and the development of the adult pigmentation pattern (Lindeman, 1929*b*). Preliminary work by the writer also indicates that the shedding of larval teeth during metamorphosis falls under the same category. Contrasted to this direct mode of hormonic influence, indirect mechanisms, involving first the degeneration or differentiation of other structures which in themselves then bring about other degenerations and differentiations, have been shown to apply to anuran metamorphosis. The transformations so initiated include the histolysis of opercular integument in the formation of the fore-leg perforations (Helff, 1926) ; the differentiation of the integumentary portion of the tympanic membrane (Helff, 1928) ; and the differentiation of the yellow fibrous region of tympanic membrane lamina propria (Helff, 1929*b*).

Summary and Conclusions

1. The origin, time and separate development of the mucous and poison glands of *Rana pipiens* have been determined as they are found, especially, in dermal plicæ regions. The results confirm those of the majority of workers on other anurans and on urodeles.

2. Larval skin transplantations designed to test the specificity of various integuments for plicæ formation were made. These included autoplastic, reciprocal transplantation of skin known to normally develop dorso-lateral or lateral dermal plicæ with integumentary grafts from the belly and back. In such cases no demonstrable histological differentiation of glandular structures was observable at the time of transplantation. During metamorphosis, the various skin grafts developed the typical macroscopic and microscopic pictures of dermal plicæ characteristic of the region from which transplantation was made. Back and belly integumentary grafts transplanted to dermal plicæ regions maintained their specific histological characteristics, failing in every case to develop dermal plicæ structure.

3. The results are discussed and the conclusion reached that the potentiality to develop dermal plicæ and their histological components is determined and fixed in certain bands of integument at a very early stage of larval growth. The differentiation of this integument to form dermal plicæ structure is probably the result of direct hormonic influence through the blood stream at a definite stage of larval involution.

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ON THE CONDUCTION OF THE CORTICAL CHANGE AT FERTILIZATION IN THE STARFISH EGG

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A number of radical changes take place in the echinoderm egg when it is fertilized, one of which is a change in the cortex which prevents the entrance of a second sperm. That the egg is not entirely passive at the time of the entrance of the sperm is well known in a number of eggs from the phenomenon of the entrance cone which flows out and partly engulfs the sperm head.

For many years there has been a difference of opinion as to whether the fertilization membrane pre-exists in the sea urchin egg before fertilization. The more recent general opinion has been that some sort of a membrane is present. A. R. Moore (1929), however, has recently presented evidence which he regards as supporting Loeb's view that this membrane does not pre-exist but is formed *de novo* at the time of fertilization. In the case of the starfish egg, however, there is no question that a definite tough membrane pre-exists which normally lifts off following fertilization. Chambers (1921) has lifted this membrane from the unfertilized *Asterias* egg with a micro-needle. It has also been shown (Whitaker, 1928) that the cytoplasm of the egg of the starfish *Patiria miniata* may be readily divided into two separate parts with a micro-needle without severing the tough outermost membrane. If an egg which is divided in this way is inseminated, both fragments can be fertilized and will develop independently within the common fertilization membrane. In this case there can be no doubt that a morphological membrane is present, and that it lifts off as the fertilization membrane, even though the properties of the membrane may perhaps change following fertilization.

As early as 1878, O. Hertwig expressed the view that it is the egg plasma itself rather than the fertilization membrane which can prevent the entrance of a second sperm. Just (1919) has pointed out that obviously the fertilization membrane rises off the egg so late after the contact of the fertilizing sperm that another and more rapid change of the cortex must be postulated to explain rejection of the second sperm.[1]

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[1] In *Arbacia* the membrane begins to lift off about eighteen seconds after contact of the fertilizing sperm, and in *Echinarachnius* after about twenty to forty seconds (from Just, 1929).

Just has further observed in *Echinarachnius* that this change, which he calls a " wave of negativity," spreads rapidly from the point of contact of the first sperm, just as the lifting of the fertilization membrane itself spreads from this point, but spreading much more rapidly so that it has covered the entire egg before the fertilization membrane has even started to rise at the entrance point of the sperm. His evidence for this " wave of negativity " is based on observations on living eggs. As soon as the tip of the first sperm has penetrated the egg, other sperm are not engulfed in the immediate vicinity of the first sperm, although they may be taken in further around the egg. As the first sperm penetrates still further, the " wave of negativity " progresses further around the egg until at the moment the sperm head has entirely disappeared into the egg, only the opposite pole of the egg can engulf another sperm. Sperm which become attached but do not enter the egg are lifted off as the fertilization membrane rises.

More recently Just (1929) and others have observed that in eggs which are in excellent condition, the cortical reaction is so rapid as to be practically instantaneous. It is practically impossible to produce polyspermy by the use of concentrated sperm suspensions. It is evident that in such innoculations the time elapsing between the contact of the first and the contact of the second sperm must be an exceedingly small fraction of a second. The spread of the cortical change in eggs in perfect condition is much too rapid to be traced by the eye. Just (1930, page 337) says, " At least for the normally monospermic ova of the marine forms which I have studied, it is certainly true that if they are in optimum condition, polyspermy is difficult if not impossible. Such ova in order to become polyspermic must undergo treatment which impairs their cortices. . . . Doubtless the very instant that contact between spermatozoon and ova is made, polyspermy is blocked."

The response of the egg to the stimulus of the first sperm involves the whole interior of the egg as well as the cortex. Flowing of the interior protoplasm immediately succeeds fertilization, as well as changes in viscosity, etc. (Heilbrunn, 1928). The interior changes may be secondary, however,—that is, consequences of changes in permeability and surface tension of the cortical region.

That the cortex performs a necessary function in admitting the first sperm, as well as in preventing polyspermy, has been shown by Just (1923) for *Echinarachnius* and for *Arbacia* and by Chambers (1921) for the starfish *Asterias*. Just burst eggs by returning them from hypertonic to normal sea water, and also by passing eggs through fine-meshed bolting silk and through lens paper to obtain endoplasmic buds or fragments bearing none of the original cortex of the egg. Chambers obtained similar fragments by means of the microdissection needle. In none of these eggs did the endoplasmic buds react to sperm or become fertilized.

The present observations add little evidence as to the nature of the cortical reaction. They are concerned with the locus or site of conduction of the spreading impulse. If it starts at a point on the cortex, it must spread either in the form of the cortical change itself, or else it must be an equilibrium shift transmitted either around the cortex, or through the egg, giving rise to the cortical change.

In a previous paper on the development of fragments of *Patiria* eggs (Whitaker, 1928), 69 cases are recorded in which two completely separated fragments lying within the same fertilization membrane-to-be were inseminated and became fertilized independently, one sperm entering each fragment. This result is almost invariably obtained when the two masses of cytoplasm are not in contact at the time of insemination. This seems to show that the impulse which spreads through the egg and results in the cortical change which prevents the entrance of a second sperm does not pass through this outermost membrane. There is no evidence of injury to the outer membrane as a result of pinching the protoplasm. It lifts off in the normal way after fertilization, although sometimes it is temporarily constricted where the needle has pressed. Additional experiments have been made in which eggs have been pinched into two cytoplasmic fragments lying within a common outer membrane, and the fragments have been allowed to flow together before insemination. In this case the treatment of the outer membrane is practically identical with that in the cases of fragments which remain apart, the difference between the two types of cases being only in the fusion or non-fusion of the protoplasm within the membrane. Only sets of eggs were used in which control tests gave 98–100 per cent fertilization. A moderately concentrated suspension of sperm was used. As a control, to test for polyspermy, five or six normal eggs were placed by means of a mouth pipette around the experimental egg, not more than several egg diameters away. Half an hour after insemination the experimental egg was transferred to a separate dish, since at the time of cleavage when the eggs are changing shape there is otherwise some danger of mistaken identity. For some time after insemination, however, the marks of cutting are clearly discernible on the experimental egg. In all cases the eggs were left in fresh sea water for about ten minutes after cutting before insemination. It has been found in a number of echinoderm eggs that this procedure permits the fragments to recover from the operation and in the case of *Arbacia* eggs, for example, it avoids polyspermy which often results from immediate insemination.

The degree of separation of two cytoplasmic fragments lying within the same outer membrane can be regulated by the extent to which the membrane is stretched with the needle, a very slight difference in the amount of stretching determining whether the fragments will flow back together or not. The eggs of *Patiria* are large, averaging about 190 or 200 microns in diameter, and they are comparatively clear, so that the grosser aspects of the nuclear and astral phenomena can usually be clearly seen in the living egg or egg fragment.

Eleven eggs were cut, after the fashion of the 69 cases already quoted, so that the two fragments were separate at the time of insemination. The control eggs lying near the experimental egg showed no polyspermy. In 9 of the 11 cases, both fragments were fertilized independently, each dividing with a single amphiaster into two normal cells (Fig. 1, *a, b, c*). In two cases one fragment only became fertilized with one sperm, the other fragment failing to become fertilized.

Twenty-five eggs were cut into two fragments and allowed to flow completely together before insemination. In 18 of these cases the fragments remained separate for one to two seconds, in three cases for more than five seconds, and in four cases for fifteen seconds to one minute. If the fragments remain apart for several minutes, a solidification of the cut surface usually prevents a complete refusion of the fragments, or in cases of fusion results in an ectoplasmic wall along the surface of fusion. In the 25 cases of complete fusion, 23 cleaved with one amphiaster into a normal two-cell stage. Apparently only one sperm had entered. There were no cases of polyspermy in the control eggs. In two cases division was by means of a triaster into three cells, apparently due to the entrance of two sperm. One of these cases of polyspermy was an egg separated for one to two seconds. The controls showed no polyspermy. The other was an egg which had been apart for more than fifteen seconds. The controls in this last case showed polyspermy, probably indicating that insemination was too heavy and that the eggs were not in the best condition.

The results show that when a sperm enters one part of an egg, usually no other sperm enters any other part of the egg which is joined by protoplasmic fusion, but another sperm does enter a part of the egg which has only the same outer membrane in common, equally well as if the parts of the egg were entirely separated and removed from one another. Apparently the cortical change which normally prevents polyspermy is not conducted through the outermost membrane, the fertilization membrane-to-be. It is conducted through the protoplasm within this membrane.

The question naturally arises: To what extent must two fragments be associated in order that the fertilization of one shall prevent the fertilization of the other? Is mere contact of the surfaces of two fragments within the outer membrane sufficient or is fusion necessary? In contrast with our extreme cases of complete separation and complete fusion, which can be identified with certainty and in which the results are clear cut, it is difficult to tell by observation in the border line cases in which the two fragments are in contact precisely what degree of fusion has taken place. Fragments which are in contact but not fused at the time of insemination usually fuse soon after fertilization. Although no statement can be made at present as to the minimum degree of contact or fusion necessary to transmit the impulse, the following experiments throw some light on the question.

Fifteen eggs were cut into two fragments which remained separate for more than a minute and then came together so that at the time of insemination they are classed as (1) touching, (2) in good contact, or (3) fused at a narrow neck, the neck being from less than ten to thirty microns through. The eggs are 200 microns in diameter. In five cases the fragments were touching. In four of these cases one sperm entered each fragment. The fragments subsequently fused, but retained an ectoplasmic partition and separated at the time of cleavage, each com-

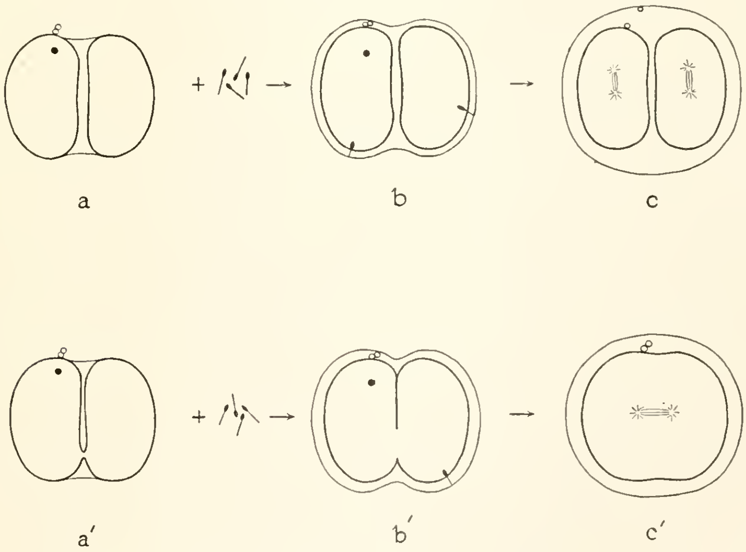

Fig. 1. Diagrams before insemination, after insemination, and just before cleavage, of eggs cut without severing the outermost membrane. (Sperm disproportionately enlarged.)

a, b, c. An egg with halves separated at time of insemination.

a′, b′, c′. An egg with halves joined by a narrow neck at time of insemination.

ponent dividing with a single amphiaster into two cells. The fifth case admitted only one sperm and divided as a whole into two cells. In three cases the fragments were in good contact, that is, about on the border between touching and slightly fused. Two of these cases had one amphiaster and divided into two cells. The third admitted a sperm into each half and divided directly into four cells. In seven cases the two halves were truly fused by a narrow neck at one end of the cut. Shortly after fertilization more complete fusion took place. In all of these seven cases only one sperm entered the joined fragments. In four of the cases normal division into two cells followed (Fig. 1, *a′*, *b′*, *c′*). In two cases the nucleus of the sperm, which had entered the non-nucleated fragment of the egg, was not able to pass through the gelated partition of the fused surfaces to fuse with the egg nucleus, and only the fragment containing the sperm nucleus divided into a normal two-cell stage. The other half separated off at the time of division but did not itself divide. In the seventh of these cases the sperm entered the non-nucleated fragment, which divided, and the nucleated fragment which apparently received no sperm divided late, involving the egg nucleus only.

These results on fragments which are only in slight contact show that a very narrow neck of truly fused protoplasm conducts the impulse. Mere contact without fusion probably does not.

SUMMARY

When eggs of the starfish are pinched into two cytoplasmic fragments which lie within the same outermost membrane (the fertilization membrane-to-be), and are inseminated while the two fragments are separate, each fragment receives one sperm. If the fragments completely fuse before insemination, only one sperm enters the fused egg. If the fragments are merely touching, each may receive a sperm. If they are joined by a very narrow fused neck of protoplasm, only one sperm enters.

CONCLUSION

The outermost membrane of the starfish egg (the fertilization membrane-to-be) does not conduct the cortical change at fertilization which prevents the entrance of a second sperm. This change is conducted by the protoplasm within the membrane.

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A STUDY OF THE EFFECTS OF MODIFICATIONS OF THE CULTURE MEDIUM UPON LENGTH OF LIFE AND FECUNDITY IN A ROTIFER, *PROALES SORDIDA*, WITH SPECIAL RELATION TO THEIR HERITABILITY

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INTRODUCTION

Extensive investigations on the Infusoria have demonstrated that these organisms, while undergoing continuous vegetative reproduction, under certain conditions " run down,"—become depressed. In many of the earlier genetic studies this progressive depression was interpreted to mean that the organisms have a definite life cycle with periods of youth, maturity, and old age, representing through the course of many consecutive generations of vegetative reproduction the usual life cycle of a single metazoan organism. The results of later investigators, however, show that in many cases the depression is partly or entirely due to unfavorable environmental conditions. Enriques, Woodruff, and others found that under adequate cultural conditions certain Infusoria can be cultivated indefinitely without decline in vitality, although the same Infusoria under poor cultural conditions exhibit a depression which may continue for long periods, becoming more and more marked as generations pass and eventually resulting in the death of most of the lines.

The inheritance of such a depression, after restoration to normal conditions, was tested by Middleton (1918) and by Jollos (1921). Middleton found that a depressed condition induced in *Stylonchia pustulata* by long continued culture at a high temperature is inherited for at least twenty days after restoration to normal temperature. Jollos produced a depression of the fission rate in *Paramecium aurelia* by subjection to calcium nitrate; he found that this lowered rate of reproduction is heritable for four months. In most other cases it has not been possible to test critically the inheritance of the depression after restoration to normal conditions, but it is commonly assumed that inheritance occurs. (For a detailed review of this type of work on the Protozoa, see Jennings, 1929.)

The present investigation was undertaken to determine how far conditions in the lower Metazoa are comparable to those in the Infusoria, particularly as to the production of depression, its increase as generations pass, and its inheritance after return to normal conditions. The Rotifera are particularly suitable for such an investigation. They resemble the Infusoria in size, habitat, and mode of life, but reproduce by means of germ cells which undergo a process of development in the production of the many-celled individual. They can be subjected to the same conditions that induce depression in the Infusoria, and the depression so produced can be studied in relation to its tendency to cumulation and its persistence after return to normal conditions.

The particular species of rotifer chosen, *Proales sordida* Gosse, has the additional advantage of reproducing exclusively by parthenogenesis, that is, from single parents, as do the Protozoa in vegetative reproduction. The complications of biparental inheritance, common to most types of germ-cell reproduction, are therefore avoided.

Insufficient feeding was the method used for producing depression: certain lines were well-fed; other lines were given insufficient food in definite ratios to the amount given the well-fed group. This method is the one used by Beers (1926) with the infusorian *Didinium*. He found that his well-fed group exhibited a high fission rate throughout his experiment; and that his inadequately-fed group showed from the first a lower fission rate, which after some time became still further depressed, this depression steadily increasing until the line encysted.

Previous experimental work on the Rotifera has been concerned chiefly with the problem of sex-determination; but there are a few papers which bear on the questions here considered. Among these are four on the effects of food control during parthenogenetic reproduction. Luntz (1926), in a critical study of the relation of food to the onset of the sexual generation in *Pterodina elliptica*, describes briefly an experiment on quantitative variation of food. Using Beneke's solution as the culture medium, and *Polytoma* or *Chlamydomonas* as the food, he varied the amount of available food by varying the number of drops used. He found that the number of offspring produced varied directly and precisely with the amount of food provided. He does not give data for successive generations; this work therefore furnishes no data on cumulative effects.

Shull (1912) and Whitney (1912*a*) carried on extensive investigations bearing upon this matter, in successive generations of the rotifer *Hydatina senta*. Both of these investigators, using various types of culture media, found that races reproducing exclusively by parthenogenesis decline in vitality, as indicated by their lowered fecundity. Whitney suggests that this may be due to "the constant environment

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of the horse manure cultures" (1912*a*, p. 343). Lambert, Rice, and Walker (1923), working under the direction of Whitney, show that this decline in vitality is due to the unbalanced diet, not to the parthenogenetic method of reproduction. None of these four investigations take up the question of the heritability of these depressions.

Other investigations, in which alcohol is used as the modifying factor, do deal with the heritability of modifications produced. Whitney (1912*c*) subjected *Hydatina senta* to alcohol and produced a decrease in fecundity which continued for two generations after return to a normal culture medium, but not longer. Noyes (1922) with *Proales decipiens,* and Finesinger (1926) with *Distyla inermis,* produced a cumulative decline in fecundity by subjection to alcohol and found that on return to normal conditions the depression continued to some degree for two generations only. (Finesinger, however, found that in weak doses alcohol acts as a stimulant and causes a slight increase in both fecundity and longevity.)

In the present study, dealing with the effects of a reduced food supply, two main questions are considered: First, do adverse environmental conditions produce the same effects in the Rotifera as in the Infusoria; that is, do they bring about a decline which increases with succeeding generations, so that the Rotifera run down as do the Infusoria? Second, if they become depressed, is this depression inherited in succeeding generations when the progeny are returned to a favorable environment?

Two characteristics, fecundity and length of life, were taken as indices of vigor. The effect of the reduced food supply on these indices was studied during the summer of 1928; the results of these experiments are presented in Part I. During the summer of 1929 the experiments, somewhat modified, were repeated; their results are presented in Part II. All of the experimental work was done in the Marine Biological Laboratory at Woods Hole.

The investigation was undertaken at the suggestion of Professor H. S. Jennings. It gives us great pleasure to express our appreciation of his advice throughout the investigation and his help in the preparation of the manuscript.

The Organism

Proales sordida is a rotifer well suited to this type of investigation. It lives in ponds and sluggish streams, and can be cultivated with ease in the laboratory in almost any infusion favorable to the growth of Protozoa; it feeds on bacteria and other minute organisms. The usual length of life is eight days, though occasionally individuals live as long as twenty-five days. Each animal produces twenty-four to twenty-eight eggs, with an observed maximum of thirty-four eggs. The adults average 300 microns in length. Reproduction is exclusively parthenogenetic. The species consists entirely of females; no males have ever been described and none have appeared during the history of our cultures even under conditions known to be favorable to male production in other rotifers. It was therefore possible, by starting with a single ancestor, to have all the animals used in these experiments members of a single clone, forming a group as genetically similar as it is possible to obtain. (For a more detailed description of this organism see Jennings and Lynch, 1928, I.)

Methods

It is not only necessary, for this type of experimentation, to have animals that are alike genetically; it is also necessary to have them as uniform as possible in other respects. Uniformity in age is particularly important, since Jennings and Lynch (1928, I) have demonstrated that the offspring of young parents have a lower fecundity than the offspring of the same parents when old. *Proales sordida* has an average life of only eight days; it begins to lay eggs about forty-eight hours after hatching. Its eggs, laid in the one-cell stage, take from twenty to twenty-four hours to hatch. A population uniform in regard to age may therefore be obtained by including in it only such eggs as are laid within a five-hour period by parents differing in age by less than twenty-four hours. To meet these conditions the following procedure was employed: In starting each experiment, several thousand eggs were removed from mass cultures and placed in oatmeal infusion in culture dishes, three hundred to a dish. Twenty-four hours later a small amount of infusion was added to each dish; at that time most of the eggs had hatched. After the lapse of another twenty-four hours, when most of the animals had begun to lay eggs, the animals were transferred to dishes of fresh culture medium. The eggs laid within the next five hours were removed and placed on two-concavity slides, one egg in each concavity in a single drop of culture medium. The animals hatching from these eggs were the first generation of experimental animals. By this procedure, eggs of approximately the same age were obtained from parents of about the same age, thus fulfilling the requirements outlined above. Throughout the remainder of the investigation, except in rare cases to be mentioned at the appropriate places, no eggs later than the third were used to give rise to a new generation.

Without exception the animals were cultivated individually on ground-glass, two-concavity slides in single drops of culture medium. The slides were kept on glass supports in nine-inch crystallizing dishes, twelve slides to a dish. Each dish was water-sealed by placing boiled spring water in the bottom of the larger portion and inverting over it the smaller portion.

The animals were examined at approximately twelve-hour intervals. In the morning each animal was transferred by means of a capillary pipette to a fresh drop of culture medium on a clean slide. In the evening the eggs which had been laid during the day were removed from the drops, but the animals were left undisturbed. The capillary pipettes were sterilized in boiling water before each transfer. Pipettes of the same size were used to place the culture fluid on the slides. The pipettes were boiled daily in distilled water and the rubber bulbs were boiled at frequent intervals.

A favorable culture medium for *Proales* is an oat infusion prepared by boiling fifteen flattened flakes (¼ gram) of rolled oats (" Quaker Oats ") for three minutes in 100 cc. of spring water, filtering this, and allowing it to stand for twenty-four hours before using (*cf.* Jennings and Lynch, 1928, I). The strength of this culture fluid may be easily varied by changing the number of flakes of oatmeal used. Such varied solutions were used in these experiments. In every case the media were prepared simultaneously in the same way, the only difference between them being the number of flakes of oatmeal used.

Part I. Experiments of 1928

In this series of experiments the following strengths of culture medium were used:

Control	Series	*C*, full-strength	fluid,	15	flakes	oatmeal to	100 cc.	spring	water
Test	"	*H*, half-strength	"	8	"	"	"	"	"
"	"	*Q*, quarter-strength	"	4	"	"	"	"	"
"	"	*S*, boiled spring water only							

These media were prepared as described above and were left exposed to the air for twelve hours before being corked.

In the preliminary experiments it was found that the animals would not live beyond the second generation in boiled spring water without oatmeal (*S* culture medium), nor in the four-flake solution (*Q* culture medium).

In the main experiment, begun July 19, 1928, and continued for three months, three groups of animals were studied: one group (*C*) was cultivated in full-strength culture medium; a second (*H*) in half-strength culture medium; and a third (*Q*) was put for its first generation into quarter-strength culture medium, into full-strength for its second generation, and then again into quarter-strength culture medium, where it remained until its death. Some individuals of the later generations of both the *H* and the *Q* groups, and their descendants, were cultivated in full strength medium (*HC* and *QC* groups).

At the start, one hundred individuals in each generation of all three groups were kept throughout their lives. But the rapid increase in coexisting generations so increased the number of animals to be examined daily that a change in procedure was necessary. In most cases the number of individuals to a generation was reduced, usually to about fifty (*cf.* Tables I and II). In other cases, not only were the members of a given generation reduced in number, but they were kept just long enough to produce the next generation, and then discarded. Such generations, of course, yielded no data on fecundity or longevity.

TABLE I

Experiment I, 1928—Showing for the successive generations (Gen.) of the two control series *C* and *M* the total numbers of eggs isolated (No.); the numbers of these that did not hatch (*N.H.*), and the numbers that hatched (*H.*); and for the latter the mean numbers of eggs laid and the mean numbers of days lived. Data presented graphically in Figs. 1 and 2.

C Series (full-strength medium)						M Series (full-strength medium)					
Gen.	No.	N. H.	H.	Mean Eggs	Mean Days	Gen.	No.	N. H.	H.	Mean Eggs	Mean Days
1	92	1	91	7.67	5.76	1	49	1	48	3.60	5.23
2	106	5	101	6.02	4.85	2	95	7	88	1.93	4.32
3	47	6	41	6.41	5.02	3	51	16	35	7.88	7.02
4	52	7	45	6.22	4.77	4	50	2	48	11.31	9.56
5	57	3	54	6.18	4.49						
6	58	8	50	3.02	3.74	9	49	1	48	9.31	11.61
7	49	8	41	1.95	3.68						
8	52	3	49	2.55	4.11	11	49	0	49	8.85	9.45
9	75	17	58	1.77	4.00						
10	56	6	50	2.74	4.18	13	42	1	41	11.58	9.17
11	51	7	44	2.40	4.38						
12	60	12	48	2.04	3.51						
13	49	4	45	1.73	3.18						
14	48	3	45	1.66	3.75						
15	49	5	44	1.36	4.15						
16	40	7	33	1.81	4.59						
17	40	4	36	2.16	4.56						
18	45	6	39	5.41	6.25						
19	49	4	45	12.17	10.06						
24	51	1	50	8.86	10.70						
26	39	1	38	12.57	10.71						
28	44	0	44	16.52	11.44						

Table II

Experiment 1, 1928. Showing for the successive generations (Gen.) of the *H* series (cultivated in half-strength medium), the *Q* series (cultivated in quarter-strength medium), and the *HC* and *QC* series (cultivated in full-strength medium), the total numbers of eggs isolated (No.), the numbers of these that did not hatch (*N.H.*), and the numbers that hatched (*H.*); and for the latter the mean numbers of eggs laid and the mean numbers of days lived.

H Series (half-strength medium)					
Gen.	No.	N. H.	H.	Mean Eggs	Mean Days
1	97	1	96	2.16	3.69
2	93	0	93	2.19	3.69
3	87	6	81	2.54	3.16
4	52	10	42	3.97	3.78
5	46	2	44	3.43	3.95
6	62	0	62	2.41	3.64
7	45	3	42	2.11	3.42
8	45	4	41	1.24	2.85
9	43	6	37	0.94	2.75
10	18	2	16	0.62	2.50
*11	7	0	7	0.14	3.00
*12	10	0	10	0.50	2.25
*13	4	1	3	0.00	1.83
HC Series (full-strength medium)					
*1(10)	14	2	12	2.50	3.95
*2(11)	27	4	23	1.04	3.19
*3(12)	23	7	16	1.62	2.90
*4(13)	24	3	21	2.90	4.57
5(14)	48	1	47	1.91	4.25
6(15)	58	10	48	2.14	4.37
7(16)	46	6	40	2.47	4.72
8(17)	45	5	40	6.45	6.83
9(18)	49	7	42	9.90	8.61
14(23)	41	2	39	8.07	11.14
16(25)	39	0	39	10.38	10.23
18(27)	49	0	49	15.14	11.15

Q Series (quarter-strength medium)					
Gen.	No.	N. H.	H.	Mean Eggs	Mean Days
1	100	0	100	0.18	2.01
†(2)	17	0	17	7.76	5.50
3	55	8	47	0.95	1.96
*4	17	0	17	2.00	3.52
5	34	2	32	1.68	3.09
6	49	1	48	1.02	2.70
7	49	0	49	0.48	2.53
*8	13	1	12	0.00	1.75
QC Series (full-strength medium)					
*1(8)	11	2	9	4.00	4.72
*2(9)	33	6	27	1.29	3.77
3(10)	34	4	30	1.30	3.30
*4(11)	35	13	22	0.90	2.95
*5(12)	20	3	17	0.64	3.26
*6(13)	9	0	9	0.77	3.88
*7(14)	3	0	3	2.00	4.33
*8(15)	2	0	2	5.50	7.75
*9(16)	8	0	8	8.12	9.18
10(17)	44	4	40	5.50	7.80
11(18)	46	3	43	6.06	8.38
12(19)	47	0	47	5.48	8.70
13(20)	54	0	54	6.98	10.24
15(22)	47	0	47	9.68	9.95
17(24)	50	0	50	14.34	11.01

†(2) Grown in full-strength medium.

* Generations comprising less than thirty animals.

General Description

I. The Control Series.

During the course of the experiment the control animals lived through twenty-eight generations, showing great variability in length of life and fecundity. This variation appeared to be correlated with the temperature fluctuations. The temperature was high when the experiment began and remained high for sixteen generations; these generations showed a progressive decline in both fecundity and longevity. The succeeding generations (17th to 27th), subjected to a lower temperature, showed a recovery which started with the first generation grown at the cooler temperature. This recovery was so swift, both fecundity and longevity increased so rapidly, that with the 20th generation it became necessary to reduce the number of animals examined. This was done by testing only occasional generations, as noted above. The number of animals in the generations not tested (generations 20, 21, 22, 23, 25, and 27) was re-

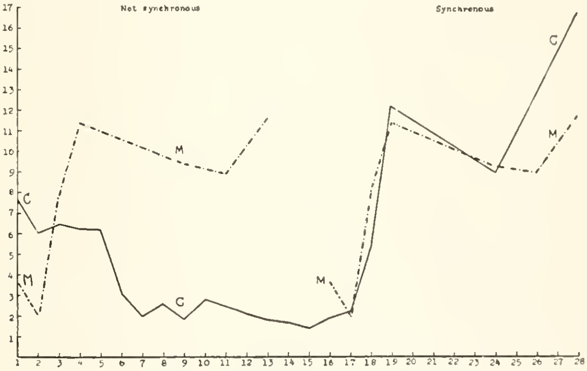

FIG. 1. Experiment I, 1928. Comparison of the two control series *M* and *C*, based on egg production; horizontal axis represents successive generations; vertical axis, mean egg production.

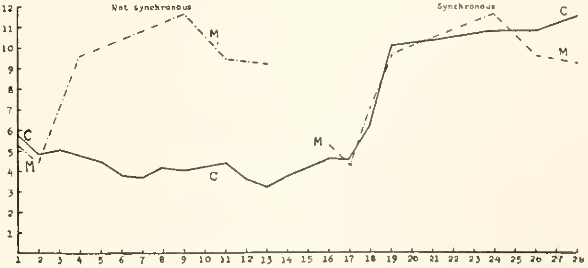

FIG. 2. Experiment I, 1928. Comparison of the two control series *M* and *C*, based on length of life; horizontal axis represents successive generations; vertical axis, mean length of life in days.

duced to thirty, and these were maintained only long enough to provide the next generation.

When the control (*C*) animals were in their most depressed condition (generation 15) a special effort was made to determine the cause of their depression by comparing them with a second series of controls, taken at this time from mass culture. This new control series (termed *M*) was treated in every way like the original control series, so that the only known difference between the two sets was the difference of date of derivation from mass culture, resulting, of course, in a diversity in duration of cultivation on slides. If duration of slide cultivation is the effective cause of depression, the early generations of *M* should not show depression; there should be a wide diversity between their records and the records of the synchronous generations of *C* (16th to 28th generations undergoing slide cultivation). If, on the other hand, some current environmental factor is the effective cause, synchronous generations of the two series should yield like data. Figures 1 and 2 demonstrate the latter condition. *C* and *M* show a similar and synchronous modality. Figure 1 is based on egg production; Fig. 2 on length of life. In both figures the record of the second control series *M* is shown in two ways; first, in relation to the records of the early generations of the *C* series, and second, in relation to the synchronous generations of *C*. It is obvious that the record of the *M* series, comprising thirteen generations, follows closely the record of the generations of the *C* series that were living at the same time; not the record of the early generations of *C*. It is therefore concluded that the cause of the depression in *C* was some feature of the current environment, probably the high temperature; and that duration of slide cultivation had had no depressive effect.

II. The *H* Series.

The series cultivated in half-strength culture medium (*H*) was consistently lower in fecundity and length of life than the controls. Consecutive generations showed some variation, but became progressively depressed, and the series died out in the 13th generation. In Table II are given the records of series *H* for every generation, showing the mean egg production and the mean length of life.

Fortunately the ultimate extinction of the *H* series was anticipated and some eggs of the ninth generation were placed in the full-strength culture medium, thus beginning a group of animals (series *HC*) in which to test recovery from depression. At the end of the experiment the *HC* group had been cultivated continuously for eighteen generations in the full-strength culture medium. It showed complete recovery from the depression (see Table II, *HC*). Generations 19, 20, 21, 22, 24, and 26 were not tested.

III. The *Q* Series.

In the preliminary experiments the animals cultivated in the quarter-strength culture medium had not survived beyond the second generation. The exceedingly low records of the first generation of the *Q* series of the main experiment indicated that they were following a similar course. Consequently, it was thought best to rear the animals of the second generation in full-strength culture medium. The records of this second generation of *Q*, cultivated in the full-strength culture medium, greatly exceeded those of the second generation of *C* animals. But the records of the third generation, cultivated in the quarter-strength culture medium, were again very low, although not as low as those of the first generation. The subsequent generations were grown continuously in the quarter-strength culture medium; they declined consistently and died out in the eighth generation.

A few eggs from the seventh generation were placed in the full-strength culture medium to test the recovery of the *Q* series. This *QC* series was continued in the full-strength solution until the end of the experiment. They improved, but their records on the whole were lower than those of the controls (*C* series) and lower than those of the *HC* series. The generations did not overlap as much in this group as in the other two groups, and at the end of the experiment, when the *H–HC* series was in its 27th generation and the *C* series was in its 28th generation, the *Q–QC* series was only in its 24th generation. Generations 21 and 23 were the only ones not tested. The record for each generation of the *Q–QC* series is given in Table II. Because of the low fecundity of these depressed animals the number of individuals in many generations was small, although all the eggs laid were kept, including in some cases eggs later than the third.

Temperature Variation

During the summer of 1927 the laboratory in which these experiments were conducted had shown fluctuations of less than two degrees (*cf.* Jennings and Lynch, I, p. 369), and it was expected that it would maintain the same constancy during the summer of 1928. Unfortunately this was not the case. The temperature of the Woods Hole region was unusually variable from July until September of that year, and the laboratory showed correspondingly greater fluctuations.

The temperature was read at twelve-hour intervals from a Maximum-minimum thermometer which was suspended above the rotifer cultures. The data thus obtained are presented in Fig. 3, which is designed to show the relation between the temperature fluctuations and the records of the control animals (*C* series). On the vertical axis the temperature is plotted in degrees centigrade; on the horizontal axis the time is plotted in twelve-hour periods, for the duration of the experiment. The upper, shaded curve (*a*) shows the maximum and minimum records for each twelve-hour period, the upper line showing the maximum, the lower line the minimum. The second curve (*b*) is a simplified form of

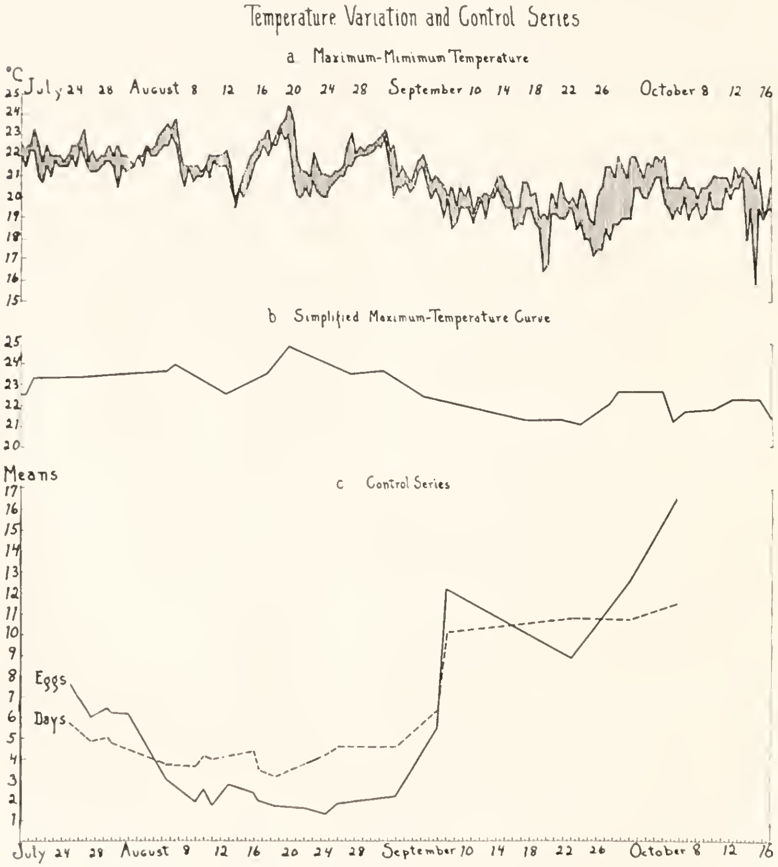

FIG. 3. Experiment I, 1928. Chart showing temperature variation and the records of control series *C*. (*a*) Upper shaded curve: the maximum and minimum temperatures at twelve-hour intervals from July 19th to October 16th; the upper line shows the maximum temperatures; the lower line shows the minimum temperatures. (*b*) Middle curve: a simplified form of the temperature curve giving only the major points of maximum temperature. (*c*) Lower two curves: the record of control series *C* for egg production (solid line) and for length of life (dashed line). The median dates of the twenty-eight generations produced from July 25th to October 5th are indicated on the horizontal axis; the mean number of eggs produced and the mean number of days lived by each generation are indicated on the vertical axis.

the upper line of the shaded chart and shows the highest records of the summer plotted at intervals of approximately one week. The third curve (*c*) shows the records of the control series (*C*) plotted on the same basis as in the foregoing curves. On the vertical scale are plotted the mean number of days of life (dotted-line curve) and the mean number of eggs (solid-line curve) for each generation. On the horizontal scale is recorded the time, as in the upper curves. The records of the controls are plotted by generations on their "Median Dates"; that is, on a date exactly midway between the date when the first animal of a given generation was isolated as an egg and the date when the last animal of that generation died.

A comparison of the three curves (*a*), (*b*), and (*c*), clearly demonstrates a close relation between the records of the control animals and the temperature. As the temperature became higher the animals became more depressed, the lowest records of the controls for the entire experiment being made immediately after the highest temperature of the summer. The animals improved when the temperature became lower. At the end of the summer, when the temperature stayed consistently lower, they gradually recovered from their depression.

Analysis of the Data

I. Method.

Inasmuch as the temperature influenced the results in such a striking way, valid comparisons of the different groups of animals could be made only if the groups to be compared lived at the same time and were thus subjected to the same temperature variations. An arbitrary method was devised to meet this condition. This method was a day by day comparison made on a percentage basis with the control series *C* as the standard.[1] The daily average record of the control series was taken as 100 per cent. The averages for the other cultures on the same day were then expressed as percentages of this. Thus if the average number of eggs produced by *H* on a given day was one-half as great as the number produced by the controls, *H* was given a rating of 50 per cent. In calculating the averages for the controls, the generations as such were disregarded, since, in an adequate medium, the number of generations cultivated on slides had been shown to be unimportant (p. 38). In calculating the records of *H* and *Q*, cultivated in the reduced media, each generation was considered separately.

The procedure for determining the percentage for each generation of an experimental group was as follows, using *H* as an example: First, the mean number of eggs for animals of a given generation of *H* was computed by days on the basis of the day they were begun as individuals. Then this mean for *H* was expressed as a percentage of the mean of *C* for the same day. In this way percentages were calculated for each day

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[1] This method was devised by Dr. Daniel Raffel of this laboratory.

Table III

Experiment I, 1928—Showing for the successive generations (Gen.) of the four test series (*H, Q, HC, QC*) the numbers of animals (No.) and the percentages of fecundity and longevity, computed as described in the text (pp. 41–43). Data presented graphically in Figs. 4, 5, 6 and 7.

H Series (half-strength medium)				*Q* Series (quarter-strength medium)			
Gen.	No.	Fecundity	Longevity	Gen.	No.	Fecundity	Longevity
		per cent	*per cent*			*per cent*	*per cent*
1	96	28.16	64.06	1	100	2.34	34.89
2	93	38.22	75.78	†(2)	17	126.57	108.65
3	81	98.48	75.40	3	47	16.67	41.55
4	42	111.79	84.91	*4	17	40.98	82.81
5	44	85.69	94.54	5	32	70.47	82.68
6	62	80.67	92.01	6	48	55.61	71.99
7	42	115.65	95.60	7	49	35.85	62.92
8	41	69.01	70.67	*8	12	0.00	41.83
9	37	39.34	65.58				
*10	16	24.72	59.22				
*11	7	65.09	74.85				
*12	10	46.88	67.60				
*13	3	0.00	50.15				
HC Series (full-strength medium)				*QC* Series (full-strength medium)			
*1(10)	12	85.58	89.67	*1(8)	9	140.74	111.46
*2(11)	23	47.75	88.16	*2(9)	27	59.49	93.08
*3(12)	16	104.80	90.83	3(10)	30	83.79	100.47
*4(13)	21	190.96	118.52	*4(11)	22	51.21	78.81
5(14)	47	123.75	98.97	*5(12)	17	42.50	82.52
6(15)	48	115.89	94.68	*6(13)	9	46.99	87.35
7(16)	40	129.71	102.81	*7(14)	3	97.05	91.99
8(17)	40	114.86	110.12	*8(15)	2	261.62	155.04
9(18)	42	80.18	84.93	*9(16)	8	87.20	97.54
				10(17)			
14(23)	39	81.83	111.33	11(18)	No controls for comparison ‡		
				12(19)			
16(25)	39	83.14	95.00	13(20)	54	79.06	93.03
18(27)	49	91.47	97.30	15(22)	47	70.49	92.62
				17(24)	50	86.45	95.91

* Generations comprising less than 30 animals.

†(2) grown in full-strength medium.

‡ Gen. 17, 18, 19 of the *QC* series were begun at a time when no control animals were begun, so comparison on the percentage basis was impossible.

included in that *H* generation. Finally, the percentages were weighted according to the number of *H* animals included in each, and the true mean of these percentages was found for each generation. Following this method, the records of fecundity and longevity of each generation of the experimental groups were calculated as percentages of the synchronous records of the *C* group. The variation of these percentages with the passing of generations is shown graphically in Fig. 4; here the vertical axis represents the percentages, the horizontal axis represents the generations, and the straight horizontal line at 100 per cent represents the standard of comparison,—the record of the *C* group.

A dependable interpretation of the data must take into consideration the numbers of animals involved; unless this number is fairly large, the sample may not be representative. In this study, generations comprising less than thirty animals have been considered of doubtful significance. These generations are indicated by asterisks in Table III. The small numbers in the later declining generations of *H* and *Q* were unavoidable, since the fecundity of the depressed animals was very much reduced and the rate of mortality was high. Fortunately, the records of these later generations are relatively unimportant, as depression is clearly demonstrated in the preceding, larger generations. But in the test of the heritability of depression in the first *HC* and *QC* generations, the small numbers available constitute a serious complication and must be taken into account fully in the interpretation.

II. Depression: (*a*) Fecundity.

A comparison of the fecundity of the three groups of animals is presented in Fig. 4. The numbers of animals in the various generations of the *H* and *Q* series and their fecundity percentages, computed as described above, are given in Table III.

Both the *H* and *Q* groups showed some variation, but in general they had a much reduced fecundity. The egg production of the first generation of the *H* series was very low; approximately 25 per cent of the *C* average. In the next six generations there was an improvement; the fecundity of the 4th and 7th generations of *H* was even higher than that of the controls. But after that time there occurred a sharp decline, and with two exceptions, each generation had a lower productivity than the preceding one. The line died out in the 13th generation.

The *Q* series followed in general the same course as the *H* series, but with an average fecundity consistently much lower throughout the experiment. In the first generation grown in the weak culture medium, the *Q* animals produced on the average less than three per cent as many eggs as the control animals. In the next few generations they improved and reached their maximum egg production in the 5th generation with an average fecundity of about seventy per cent of the record of the controls. From that time on there was a steady decline, each generation having a lower fecundity than the previous one. The maximum depression was reached in the 8th generation with the complete dying out of the line.

Subjecting *Proales sordida* to culture media of reduced strength for many successive generations is thus shown to cause a decrease in fecundity similar to that observed in other rotifers in adverse environmental conditions, and similar in many respects to the lowering of fission rate noted in poorly-fed Infusoria.

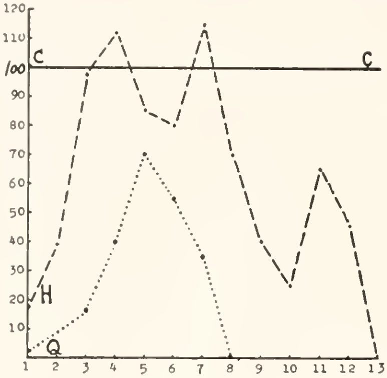

FIG. 4. Experiment 1, 1928. Depression curve based on egg production. Horizontal axis represents successive generations; vertical axis the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard. In *H* the culture medium is half the strength of that in the controls; in *Q*, it is one quarter of that strength.

(*b*) Longevity.

There is very little in the literature on the effect of adverse conditions on longevity. Most of the environmental studies on the Rotifera present data dealing only with the effect on fecundity, considered as an index of vitality. For this reason the longevity data of these experiments are of special interest. Those for Experiment 1 are presented graphically in Fig. 5 in the manner already described in the consideration of fecundity. Table III gives a résumé of the records for every generation.

The effect on the length of life of the two underfed series was greater than the effect on fecundity, discussed in the last section; this is especially true of the *H* series. Neither the *Q* series nor the *H* series ever equalled in average length of life the record of the *C* group; and the records of the three coexisting groups fall in precisely the same order, throughout their history, as that of the strengths of their culture media.

In the *H* group, the first generation lived, on the average, about sixty-five per cent as long as the contemporaneous members of the *C* group. Generations 2 to 7 showed an increase in relative length of life, rising in the 7th generation to about ninety-five per cent of the *C* record. Generation 8 showed a sharp decline which continued with two exceptions, until the 13th generation, when the line died out.

In the *Q* group the average life of the first generation was about thirty-five per cent of that of the controls. The next few generations showed an improvement, and in the 4th generation the average longevity of the *Q* line practically equalled that of the 4th generation of the *H* group. From then on, however, there was a rapid decline; the line died out in the 8th generation.

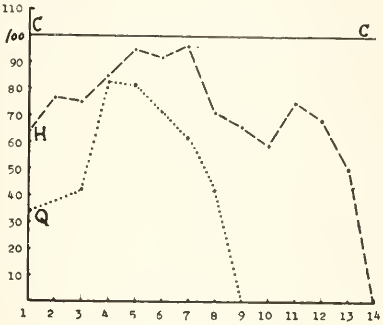

FIG. 5. Experiment I, 1928. Depression curve based on length of life. Horizontal axis represents successive generations; vertical axis represents the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard.

These results are diverse from those obtained on the effect of alcohol by Noyes for *Proales decipiens*, and by Finesinger in most of his experiments on *Distyla inermis*. They did not find that the length of life was appreciably decreased by subjection to alcohol, even though the depressive effect on fecundity was very marked. Also, Finesinger found that in very weak doses of alcohol, both fecundity and longevity were increased. In the experiments here reported, on *Proales sordida* cultivated in inadequate media, both fecundity and longevity were strikingly decreased.

In general, then, we may say that *Proales sordida,* when grown in dilute culture media (quarter or half as strong as the optimum), and under variable temperature conditions, after an initial immediate depression, tends to adjust itself for a few generations and pursue an upward course, but eventually becomes progressively depressed and dies out completely. Individuals grown in a quarter-strength solution have a lower fecundity and longevity than those grown in a half-strength medium, and in turn, individuals grown in a half-strength medium, for the most part, have a lower fecundity and longevity than those grown in a full-strength solution. In other words, the degree of depression and the speed with which it occurs are, to a large extent, dependent on the strength of the culture medium.

III. Recovery.

The question of the heritability of induced modification is of very great interest; it forms the problem of chief concern in these experiments. To test this, some of the progeny of each of the depressed groups were placed in control medium (full-strength) and their successive generations were continuously cultivated in this medium. These "recovery groups" (*HC* and *QC*) were begun with eggs from the ninth generation of *H* and from the seventh generation of *Q*. In the curves showing the records of these groups (Figs. 6 and 7) the first genera-

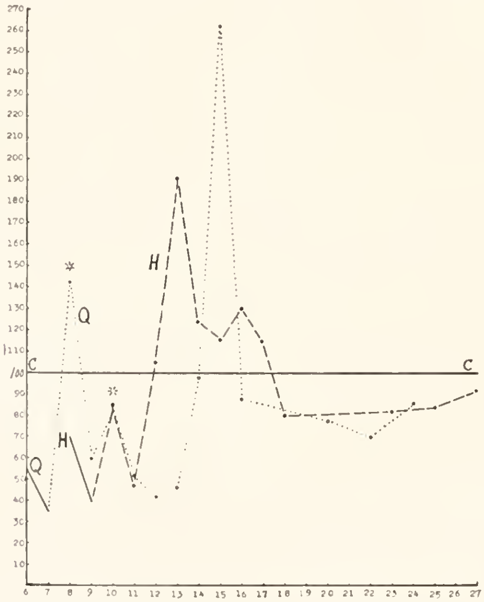

FIG. 6. Experiment I, 1928. Recovery curve based on egg production. Horizontal axis represents successive generations; vertical axis represents the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard. Asterisks indicate the generations in which return to normal medium occurred.

tions are marked with an asterisk. All computations were made on the percentage basis described above, using the record of the controls as the standard for comparison. On the vertical scale are plotted the percentages; on the horizontal scale the number of generations. The record of the controls is indicated by the solid straight line at 100 per cent. The short solid lines preceding the dashed *HC* line and the dotted *QC* line represent the records of the generations of depressed animals (*H* and *Q*) from which the recovery groups were begun. For the detailed data on fecundity and longevity, and the numbers of animals comprising each generation, consult Table III.

(*a*) Fecundity.

At the time when the return to normal medium was made, the *H* series had an average fecundity about forty per cent of that of the control animals. As is shown in Fig. 6, the first generation in the normal medium (generation 10, marked with an asterisk) showed a considerable increase in productivity, rising to about eighty-five per cent. The next *HC* generation (generation 11) fell to almost forty-seven per cent again. The third *HC* generation (generation 12) improved markedly, with an average egg production slightly higher than that of the control series; and the next five generations, though showing a considerable fluctuation, maintained an average fecundity consistently above that of the controls. In the 9th generation in normal medium (18th generation of cultivation) the *HC* group had an average fecundity of 80 per cent and stayed at about that level for the remainder of the experiment.

The animals from the *Q* line which were placed in control medium (full-strength) also showed great variation in fecundity. Ten of the twelve *QC* generations had a fecundity lower than that of the controls. The other two (first and eighth *QC* generations) showed a fecundity much higher than that of the controls. The first *QC* generation showed an increase from 36 per cent (the record of the preceding *Q* generation, cultivated in quarter-strength culture medium) to about one hundred and forty-one per cent, exceeding the control record by a wide margin. The eighth *QC* generation (comprising only two animals) rose to 262 per cent, more than doubling the record of the controls.

For a correct evaluation of the significance of these very irregular records of the *HC* and *QC* series, the numbers of animals in the various generations (given in Table III) must be taken into account. With at most two exceptions the early generations of recovery are composed of so few individuals as to render the significance of their data doubtful. As far as they go, they indicate that recovery of egg production is immediate and complete in the first generation grown in normal culture medium.

(*b*) Longevity.

On the whole, in both test lines, variation in length of life after the return to normal culture medium was much less than variation in egg production. The data are shown in Fig. 7, plotted on the percentage basis as earlier described. The first generations in full-strength culture medium are indicated by asterisks.

The average length of life of the first generation of the *HC* series was considerably greater than that of the preceding *H* generation. This increase in longevity was continued for several generations, the 4th generation of recovery (13th) exceeding the record of the controls. From that point on, there was considerable variation, the average length of life of the *HC* series fluctuating around the record of the controls. Here, as in the case of fecundity, the fact that the early generations comprised only small numbers of animals makes the significance of their data doubtful.

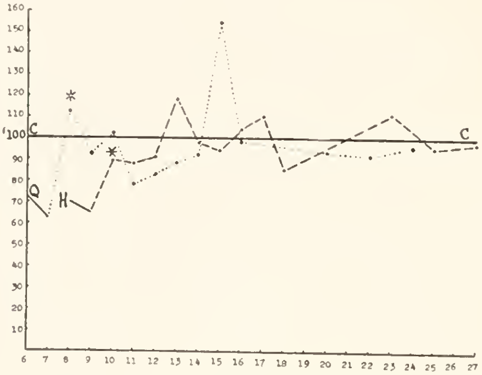

FIG. 7. Experiment I, 1928. Recovery curve based on length of life. Horizontal axis represents successive generations; vertical axis represents the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard. Asterisks indicate the generations in which the return to normal medium occurred.

In the *QC* series, the average length of life of the first generation exceeded the record of the controls, indicating that recovery was immediate and complete. In generations 2 and 3 (9 and 10) the longevity of the *QC* series remained very close to that of the controls; but after that, except for one generation (15th) the average was slightly lower than that for *C*. Here, also, the fact that only one (generation 10) of the first nine generations of recovery in this group was significant from a statistical point of view makes the results unreliable. However, the indication is that the decreased length of life produced by insufficient food is not inherited when the progeny are placed in an adequate environment.

Discussion

The results of these experiments show that the Rotifera do respond to an unfavorable environment as do the Infusoria; they become progressively depressed, show a gradual lowering of vitality, and eventually die out. In the adverse condition used, an inadequate culture medium, successive generations show this graded decline in both fecundity and longevity.

The results bearing on the question of the heritability of induced modifications, however, are not decisive, owing to their great irregularity. It seems probable that this irregularity was largely due to the extreme temperature variation and to the inconsiderable number of animals available in the critical generations. To control the temperature, and in the light of the experience gained to test again the heritability of the depression, a second series of experiments was carried out. An account of these follows.

PART II. EXPERIMENTS OF 1929

In 1929 the experimental work was conducted in a constant temperature room where fluctuations were very slight (20° to 22° C.). Variations from day to day were usually less than half a degree, and omitting the last week of the experiment when considerable fluctuations occurred, the total variation for the summer was less than two degrees.

Preliminary tests were made to find out whether cultivation under uniform temperature necessitated any change in experimental procedure. It was found that under constant temperature conditions rotifers reared in the culture medium used for the controls in the former experiments (15 flakes of oatmeal to 100 cc. spring water) had a very low fecundity and longevity and appeared to be starved. Consequently, solutions of 16, 20, 24 and 32 flakes of oatmeal to 100 cc. of spring water were tested. The 32 flake fluid was found to be the most satisfactory.

In Experiment II, therefore, the following culture media were used:

Control	Series	*C*, full-strength	fluid,	32	flakes	oatmeal	to 100 cc.	spring	water
Test	"	*H*, half-strength	"	16	"	"	"	"	"
"	"	*Q*, quarter-strength	"	8	"	"	"	"	"

In general these media were prepared as earlier described under "Methods," except for the fact that the culture fluid was corked at the end of three hours and placed in the constant temperature room, instead of being corked at the end of twelve hours and subjected to variable temperature. It will be observed that in Experiment II the half-strength and quarter-strength culture media were twice as strong as the corresponding media of Experiment I, and that the fluid for the control series was more than twice as strong as the full-strength medium formerly used. In all probability, the fact that the culture fluid was exposed to the air for such a short time (three hours) explains the necessity for increasing the number of flakes of oatmeal used.

General Description

This experiment was begun August 11, 1929, with a single series of control animals which were cultivated on slides for three generations before the test groups were started. These test groups, again called *H* and *Q*, were begun with some of the eggs laid by the third generation of this control series. In the 4th generation, therefore, there were three series of animals: the controls (*C*) cultivated in thirty-two flake fluid; the *H* series cultivated in sixteen-flake fluid; and the *Q* series cultivated in eight-flake fluid.

In general, Experiment II was conducted in the same way as Experiment I, dealing again with the effects on fecundity and longevity produced by continuous cultivation for many generations in weak culture media. When the animals had shown a marked decrease in productivity and length of life, the heritability of this environmental modification was tested by placing progeny from both test groups in the control medium of thirty-two flakes, and cultivating them in this full-strength medium for several successive generations.

Analysis of the Data

Since the corresponding generations of all three groups of animals occurred at the same time (except during the last part of the experiment, when the control line was sufficiently in advance of the other two groups to produce one more generation), and since the temperature conditions were satisfactorily controlled, there was no reason for comparing the groups of animals on any basis except that of corresponding generations. The results of the experiment are shown on this basis in Figs. 8 to 11. In these curves the horizontal axis represents the number of generations, using 1, 2, 3 for the preliminary series, then re-numbering 1, 2, 3, etc., beginning with the first generation to comprise three groups of animals. The vertical axis represents either the mean number of eggs or the mean length of life. The data for these curves will be found in Table IV.

I. Depression.

In this experiment, conducted under constant temperature conditions, and with stronger media than those used in Experiment I, the test lines, *H* and *Q*, did not die out with the passage of generations. When the

Table IV

Experiment II, 1929—Showing for the successive generations (Gen.) of each of the five series of the second experiment (Control, *H*, *Q*, *HC*, *QC*) the numbers of eggs isolated (No.), the numbers of these that did not hatch (N.H.), and the numbers that hatched (H.); and for the latter the mean numbers of eggs laid and the mean numbers of days lived. Data presented graphically in Figs. 8, 9, 10, 11.

Control Series (full-strength medium)					
Preliminary					
Gen.	No.	N.H.	H.	Mean Eggs	Mean Days
1	24	0	24	20.75	10.00
2	21	0	21	16.95	8.66
3	39	1	38	17.28	8.89
Principal					
1	48	1	47	13.02	7.67
2	52	0	52	12.34	8.17
3	41	3	38	14.00	9.13
4	40	1	39	15.38	9.01
5	40	0	40	13.87	8.17
6	54	3	51	14.31	7.93
7	30	3	27	10.48	7.62
8	34	0	34	9.23	7.26
9	30	2	28	9.53	6.94
10	35	4	31	10.19	6.30
11	29	0	29	8.13	5.79
12	28	0	28	6.46	5.32
13	30	7	23	6.26	6.19
14	39	12	27	9.03	7.35
15	32	10	22	8.59	9.00
H Series (half-strength medium)					
1	50	1	49	9.97	7.34
2	52	1	51	8.25	7.58
3	43	2	41	5.63	6.76
4	39	0	39	6.74	7.55
5	40	4	36	6.97	7.09
6	43	2	41	6.04	6.71
7	51	5	46	5.21	6.71
8	42	4	38	5.63	6.82
9	41	1	40	6.22	6.13
10	39	3	36	4.36	5.97
11	42	3	39	3.82	5.33
12	42	9	33	3.54	6.12
13	36	6	30	5.53	6.71
14	27	4	23	8.52	8.50
HC Series (full-strength medium)					
1(13)	38	4	34	12.50	8.67
2(14)	29	1	28	8.17	8.26

Table IV—*Continued*

Gen.	No.	N.H.	H.	Mean Eggs	Mean Days
Q Series (quarter-strength medium)					
1	41	0	41	5.19	5.74
2	38	2	36	2.75	5.13
3	43	7	36	1.33	3.68
4	31	0	31	4.29	6.51
5	51	1	50	5.46	6.59
6	48	4	44	3.00	5.30
7	40	1	39	2.66	5.66
8	40	4	36	1.83	5.00
9	42	3	39	1.64	4.24
10	62	11	51	1.39	4.00
11	63	9	54	2.33	4.93
12	54	5	49	3.51	5.72
13	28	0	28	2.10	4.60
14	34	4	30	1.53	5.05
QC Series (full-strength medium)					
1(9)	24	1	23	9.43	6.04
2(10)	50	9	41	8.85	6.48
3(11)	41	16	25	6.72	6.18
4(12)	39	2	37	12.91	8.29
5(13)	27	4	23	10.39	7.50
6(14)	31	4	27	6.03	7.14

experiment was concluded they had lived fourteen generations and, while showing some depression, they were reproducing well enough to make it seem probable that they would live many more generations, if not indefinitely.

(*a*) Fecundity.

Figure 8 presents graphically the data on egg production. It shows that in general the records of the three groups of animals ran parallel to each other, the half-strength (*H*) series regularly lower than the control series, and the quarter-strength (*Q*) series regularly lower than the *H* series. At first, the difference between the three groups was very great, but at the end of the experiment this difference was much less, especially between the *H* and *C* series. The control series showed considerable variation, but its average fecundity gradually decreased as the experiment progressed. The *H* line declined rapidly for the first three generations, and from then on continued to show a fairly constant though much less extensive decline until the 13th and 14th generations. In these last two generations the *H* line showed a marked improvement, almost equalling the average of the controls. The *Q* line likewise declined rapidly when first subjected to the reduced culture medium, showing in the third generation the maximum effect on fecundity, when the average egg production fell to 1.33. The average fecundity stayed consistently low for the remainder of the experiment although showing considerable variation. It showed no improvement in its final generations such as the *H* series showed; the 13th generation had an average fecundity of only 2.1 eggs and the 14th generation of only 1.5 eggs. The individuals were small and thin.

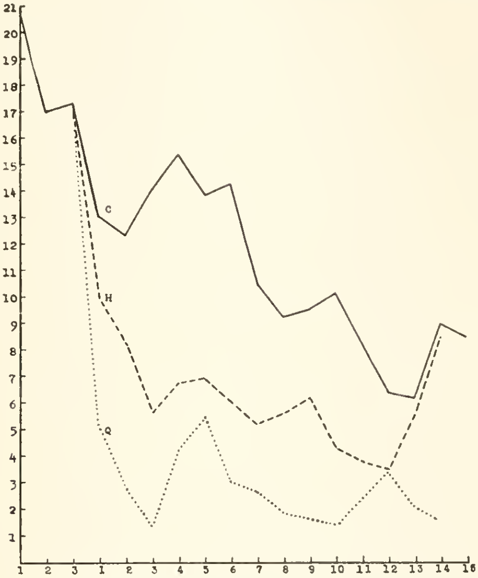

FIG. 8. Experiment II, 1929. Depression curve based on egg production. Horizontal axis represents successive generations; vertical axis, mean egg production.

Thus in this experiment, as in the former one, decreased food is associated with a decrease in fecundity, the most marked decrease occurring in the first three generations.

(*b*) Longevity.

The effects on longevity are presented in Fig. 9. The *H* series showed a moderate and gradual decline for eleven generations, but then began to improve; in the 12th, 13th, and 14th generations its average longevity exceeded that of the controls of the corresponding generations.

In the *Q* line the effect of the adverse condition was more marked, and for the first few generations the average length of life declined rapidly, reaching its lowest point in the third generation. From that time on the *Q* line showed considerable variation but remained consistently below the other two groups except in the 12th generation, when the average length of life was slightly above that of *C*, but did not equal that of *H*.

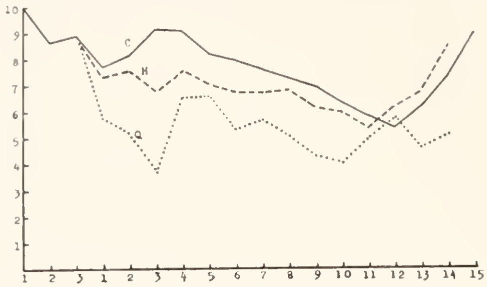

FIG. 9. Experiment II, 1929. Depression curve based on length of life. Horizontal axis represents successive generations; vertical axis, mean length of life in days.

II. Recovery.

The *H* recovery series was not begun until very late in the experiment. We wished to test progeny from the most depressed generation and for that reason delayed beginning this series in anticipation of further depression in *H*, which did not occur. The *HC* series was finally started with eggs from the 12th *H* generation, a short time before the experiment terminated, and so was carried through only two generations. The *Q* recovery series was begun with eggs from the 8th generation in the quarter-strength medium. This *QC* line was continued in normal medium for six generations, when the experiment ended. The data on these *HC* and *QC* lines is presented in Figs. 10 and 11. Asterisks indicate the generations where the return to normal medium occurred.

(*a*) Fecundity.

While the *H* line in the 12th generation had an average egg production of 3.54, their progeny which were returned to the normal medium had an average of 12.5, exceeding the average of the controls by 6.24 eggs. The second (the last) *HC* generation fell to an average slightly below that of the controls.

The *Q–QC* series increased from an average of 1.83 eggs in the 8th generation in the quarter-strength medium to 9.43 eggs in the first generation in control medium, practically equalling the controls of that generation. They remained slightly below the controls for two generations and then surpassed them by an enormous margin in the 4th generation (twelfth under cultivation). After this they again declined to a point below the controls. The records of these two lines certainly provide conclusive evidence that the effect of insufficient food on fecundity is not inherited.

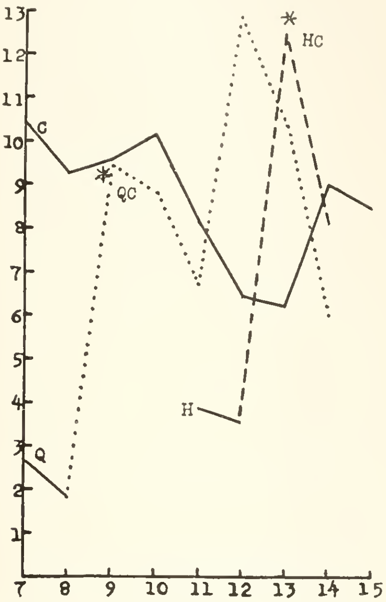

Fig. 10. Experiment II, 1929. Recovery curve based on egg production. Horizontal axis represents successive generations; vertical axis, mean egg production. Asterisks indicate generations in which return to 100 per cent medium occurred.

(*b*) Longevity.

Figure 11 clearly shows that animals from both the test series, when placed in full-strength culture medium, recovered a normal longevity at once, their records even exceeding that of the control animals. The record of the first *HC* generation exceeded by the same amount the record of their parents (the last *H* generation) and the record of the corresponding generation of the *C* series, and fell only slightly in their second and last generation.

The longevity of the first generation of the *QC* line, grown in the full-strength medium, almost equalled the record of the controls; the four generations following exceeded the controls, and the sixth (the last) generation was only very slightly below the controls.

What has been said concerning the inheritance of decreased fecundity is likewise true for longevity. Animals whose length of life has been altered by long cultivation under adverse conditions produce progeny that show a normal longevity when grown in a favorable environment. Depression produced in this rotifer by the action of an inadequate culture medium lasts only as long as the stimulus which produces the depression is present. This result differs a little from the results of Whitney (1912) and Noyes (1922), who found that the depression produced by the action of alcohol persisted for two generations after removal to a favorable medium.

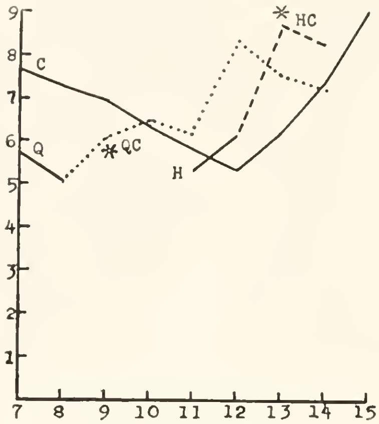

FIG. 11. Experiment II, 1929. Recovery curve based on length of life. Horizontal axis represents successive generations; vertical axis, mean length of life in days. Asterisks indicate generations in which return to 100 per cent medium occurred.

Mechanism of the Effect

As to the precise way in which the environment operates to produce depression, we have no evidence. It may be that in a medium deficient in food, the rotifer grows at the expense of its own productivity; that in the ordinary metabolic processes it uses up part or all of the yolk stored in the vitellarium, making the production of many eggs impossible, thus bringing about a depression in fecundity. This process may be correlated with the length of life of the organism to the extent that when all the stored yolk has been used up in metabolism, the animal has no more reserve on which to depend and consequently dies, thus bringing about a lowering in longevity. Or, on the other hand, in such a weak food medium the animal may use all the food it receives for its own body metabolism and not have enough left to produce yolk for many eggs, causing a reduction in fecundity but not in longevity, the animals having sufficient vitality to continue living for the usual length of time if they do not use part of their energy in egg production.

It is possible that the amount of stored yolk is reduced in ill-fed animals, and that the eggs are smaller. If this were a cumulative process, the eggs of consecutive starved generations becoming progressively smaller, containing less and less yolk, the lines would eventually die out. Measurements need to be made to determine whether there is any size difference between the eggs of normal parents and the eggs of depressed parents. As far as we could determine by superficial observation, the eggs of all generations of all three groups were normal in every way.

No measurements were made on the animals in our experiments, but some of the observations on comparative size and general appearance are of interest. In the second experiment, particularly, the difference in appearance between animals belonging to different lines was very striking. The controls were large and healthy in appearance from the first; the animals of the *H* series very soon became slightly smaller than the controls, and quite transparent; the *Q* individuals were extremely small and thin. For several generations one could recognize at once to what line an animal belonged merely by its size and type. As the experiment proceeded, however, the *H* and *Q* animals improved in appearance to such an extent that in the later generations they seemed as healthy and normal as the control animals. In the last two generations some of the control animals looked bloated and heavy, and died containing dark masses of material, and often one or two or even three well-developed eggs which they had been unable to lay.

In both of the experiments, animals were observed with internal eggs which they were unable to eject; in numerous cases the egg developed within the female and hatched within her body after her death. Some of these young finally escaped from the parental corpses and lived thereafter as normal individuals, apparently not differing from animals that had hatched normally. A single instance was observed where two such young hatched out within the same female and later became liberated. This behavior was not confined to one group or one generation, but instances were observed in *C*, *H*, and *Q*, in the later generations of both experiments.

SUMMARY AND CONCLUSIONS

This paper is a study of depression and recovery in the parthenogenetic rotifer, *Proales sordida* Gosse, showing the response of the organism to various dilutions of culture medium. All the animals used were members of a single clone and hence were genetically alike. Oatmeal infusions of half and quarter-strengths, as compared with the full-strength medium used for the control animals, were employed. The investigation consisted of two series of experiments conducted separately one year apart, together with some supplementary procedures. It was found that with continuous parthenogenetic reproduction, rotifers in the reduced strengths of culture media become depressed, decline in vitality with the passage of generations, showing a much lowered fecundity and longevity. Under conditions of variable temperature this decline is cumulative and results in the ultimate death of the lines. Under constant temperature conditions the animals show a cumulative decline in the first few generations, reaching a low level of fecundity and longevity, where they remain without further depression. The degree of depression and the speed with which it occurs depends on the strength of the medium employed. This depression produced by means of an environmental agent is similar in many ways to that produced by the action of adverse conditions in various Infusoria and in other rotifers. However, in contrast to the results obtained by others in studies of the Rotifera, longevity as well as fecundity is affected by the environmental agent.

The progeny of the depressed animals recover their normal fecundity and longevity at once in the first generation placed in a favorable medium. Apparently the food deficiency does not affect the germ cells so as to produce a heritable modification.

The protozoan constitution seems to be extremely sensitive to environmental modifications and becomes changed to such an extent that the alterations are passed on to the progeny of later generations. Some of these changes are adaptive, so as to cause an increase in resistance to certain agents, while others (such as those resulting from food insufficiency) are degenerative and result ultimately in the extinction of the race. The situation in the Rotifera, as apparently in the other Metazoa, is quite different. Though in Rotifera it is possible to produce and maintain depression for a long period of time, and though the depression becomes greater in later generations, it disappears at once, or within one or two generations, upon restoration to normal conditions. The complete agreement in this respect of the results of the present study with those of Whitney, Noyes, and Finesinger on the injurious effects of alcohol goes far to establish this as a definitive conclusion for the Rotifera. Injurious conditions acting on the individuals of many successive generations and producing a progressive decline, do not alter the germinal materials carried by these individuals.

It is possible that this difference between the Rotifera and the Protozoa may be related to the differences in the details of their reproductive processes. In the Protozoa the body divides into two equal parts; each part becomes a new individual. In *Proales*, as in most Metazoa, a smaller piece separates from a larger. The larger piece is called the parent; the smaller the egg. This smaller piece goes through a twenty-four-hour-long process of development, including numerous cell divisions, into a new *Proales* individual. It does not seem obvious *a priori* that this difference in detail must result in diverse genetic behavior. These experiments on the heritability of an environmental effect indicate that they do.

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THE RÔLE OF THE BASAL PLATE OF THE TAIL IN REGENERATION IN THE TAIL-FINS OF FISHES (*FUNDULUS* AND *CARASSIUS*)

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Experiments on the tails of fishes from a standpoint of morphogenesis have been performed by Morgan (1900, 1902 and 1906), and by the writer (1929). A review of the literature may be found in Nabrit (1929).

The writer suggested in the previous paper, after comparing the findings of Harrison (1918) and Detwiler (1918) in *Amblystoma* with the results of his experiments in fishes, that a possible similarity existed between the production of limbs in *Amblystoma* and tail-fins in fishes. Each one seems to be an independently differentiating mesenchymal system.

It was further concluded that the rate of regeneration from cut surfaces in the tail-fins of fishes is regulated by the cross-sectional area of the fin rays exposed.

Broussonet (1786), Morgan (1906), and Morrill (1906) agreed that ray stumps must be left for regeneration to take place. Morrill suggested that regeneration does not take place when the remaining stumps are too small. This point was left open for further investigation by the writer and is considered in this paper.[1]

The experiments were carried out during the summer of 1929 at the Marine Biological Laboratory at Woods Hole, Massachusetts, and during the winter of 1929–1930 at Morehouse College. At Woods Hole, *Fundulus heteroclitus* was used and at Morehouse College, the goldfish, *Carassius auratus*. In both cases, adult animals were used.

Rays were carefully picked from the tails by fine forceps. The tail and the rays were examined under a binocular microscope to make sure that no parts of the rays were left embedded in the tails.

After regeneration was complete, examinations were made upon the living and then upon the fixed tails.

In three weeks after removing the fin rays, undifferentiated mesen-

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[1] I wish to express my sincere thanks to Professor J. Walter Wilson of Brown University for his very helpful suggestions and criticisms in this investigation.

chyme had filled in the gap made in the tails. In from four to six weeks, rays appeared in the regenerated mesenchyme (*a*, Figs. 1 and 2). These rays begin at the basal plate and differentiate distally. They segment and may branch as any other rays in the tail, though some rays do not branch (*b*, *c*, Figs. 1 and 2). If they branch at all it is usually during the first two months. The anterior knobs or proximal ends of the rays are completed in from five to six months (*e*, *e′*, Figs. 1 and 2). The proximal ends are produced on both sides of the basal plate.

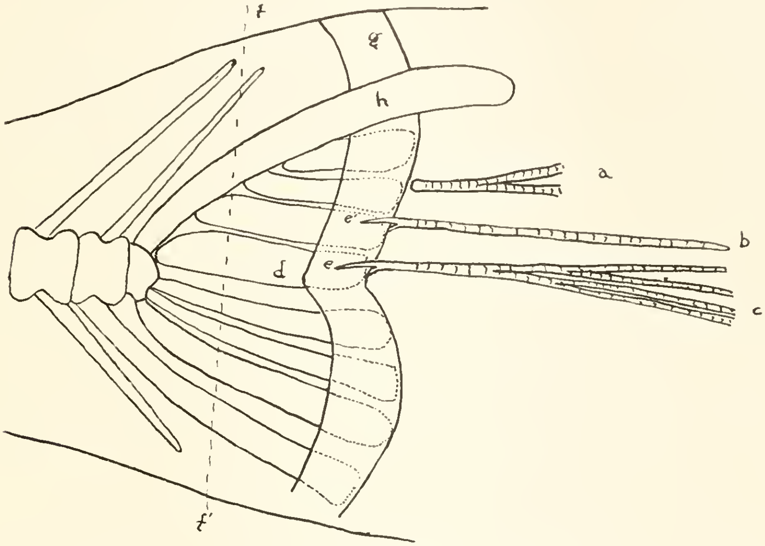

FIG. I. *Carassius.* Diagrammatic sketch of the base of the tail.
a. A newly regenerated ray (six weeks).
b. A ray six months after it started regeneration. It did not branch.
c. A regenerated ray after six months.
d. Basal plate.
e and *e′*. Proximal end of fin ray.
ff′. Region of a cut anterior to proximal end of fin rays.
g. Articulating region of basal plate.

In the embryonic development of the tail in *Fundulus*, the rays streak out from a common mesenchymal plate. They begin to appear between the ninth and thirteenth days. From two to four appear at a time, dorsally and ventrally placed in respect to the previously formed rays.

When a cross-cut is made in the tail anterior to the proximal knobs of the fin rays (*ff′*, Figs. 1 and 2), regeneration does not readily take place. This led to the conclusion that the ray stumps must be left in the tail for regeneration of the tail to occur.

When rays are picked out of the tail, they readily regenerate; the rate, however, is slower than it would be if ray stumps were present. It would appear, therefore, that no parts of the rays are necessary for regeneration.

New rays do not begin to appear at their original most proximal point, but at the distal end of the basal plate. Physically this is necessary unless two separate anlage are developed for each ray, as each ray has a component on each side of the basal plate.

When a cross-cut is made anterior to the proximal knobs of the fin rays, the cut also includes the anterior articulating portion of the basal plate. In such a case the delay in regeneration would be so great that the animal would be cast aside, even if, as is usually the case, the flesh did not slough off and lead to death.

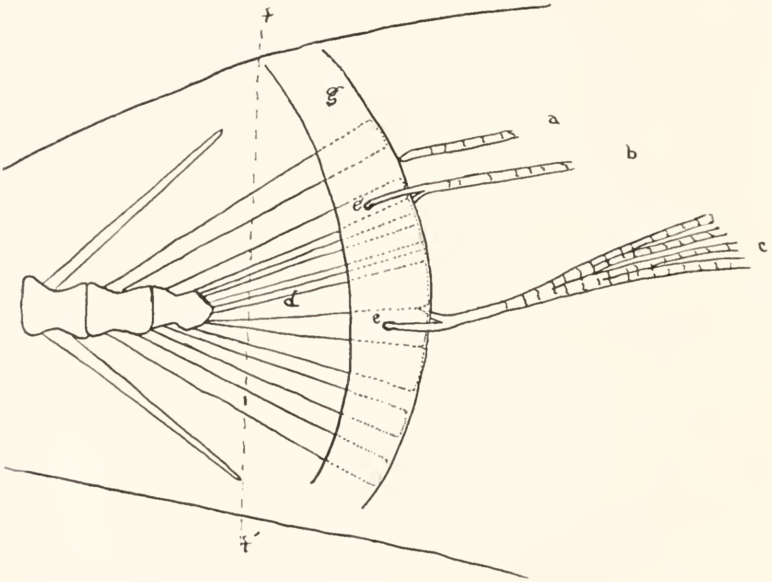

FIG. 2. *Fundulus.* As above.
a. New ray, three and one-half weeks.
b. A ray, six and one-half weeks.
c. A normal ray.
d. Basal plate.
e and *e'*. Proximal end of fin ray.
ff'. Region of a cut anterior to proximal end of fin rays.
g. Articulating region of the base of tail.

A cut ray will regenerate those parts distal to the level of the cut. An anterior knob regenerates the entire ray. Axial heteromorphosis on the knob has not occurred. But when an anterior knob is severed or removed from the distal stump, axial heteromorphosis occurs in the distal portion. When a ray is picked out, on the other hand, the new distal portion appears at the end of the articulating portion of the basal plate. Now instead of getting axial heteromorphosis with this distal portion, there is added a normal anterior knob. The visible difference in these two cases is that this new ray is in connection with the basal plate. It thus seems evident that the basal plate gives rise to the parts distal to it, or induces their development. This suggestion gains further support from the fact that the articulating part of the basal plate is shaped like the tail in *Carassius* and in *Fundulus,* bilobated in the former and rounded in the latter, and may still retain the original formative influences which cause the differentiation of the fin rays and hence the size and form of the tail.

It seems that a further similarity is demonstrated between the morphogenesis of tail-fins of fishes and the *Amblystoma* limbs. The articulating plate, like the girdle, may give rise to parts distal to it. In regeneration as in embryonic development, the basal plate gives rise to or induces the development of rays. The rays, like the limbs in *Amblystoma,* and the basal plate, like the girdle, belong to a self-differentiating mesenchymal system.

Summary

1. By picking out the fin rays instead of cutting anterior to them, it is shown that ray stumps are not necessary for regeneration in the tail-fins of fishes.

2. When the stumps are removed it seems that the new rays appear under the influence of the articulating portion of the basal plate of the tail.

3. From the embryological development and mode of regeneration a similarity is demonstrated between morphogenesis of limbs of *Amblystoma* and the tails of fishes, each being a self-differentiating mesenchymal system.

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RELATIVE RESISTANCE OF FOURTEEN SPECIES OF PROTOZOA TO THE ACTION OF *CROTALUS ATROX* AND *COBRA* VENOMS

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Essex and Markowitz (1930) have offered data which lend support to the conception that *Crotalus* venom is a non-specific protoplasmic poison that affects protoplasm wherever it comes in contact with it. This is in contrast with the view that snake venom is a complex of various constituents, each specific for certain kinds of cells. In a recent publication (1930) I have presented data, on eleven species of Protozoa, which indicate a wide difference in the degree of susceptibility to the effect of *Crotalus atrox* venom. Some species were not affected by the concentration of the venom used (0.00025 gram of dry venom per cc. of medium), while some were killed quickly. No attempt was made, however, to determine whether the races of Protozoa found to be resistant would be affected by higher concentrations of the venom. In the light of the conclusions of Essex and Markowitz concerning the general nature of the poisonous effects of *Crotalus* venom it has seemed advisable to continue the investigation of the effect of venoms on Protozoa to determine if any species is wholly resistant to their destructive action. The investigation is continued also as a phase of a more extended study, which is in progress, of the resistance which races of Protozoa acquire to venoms.

For each of the fourteen species of Protozoa used in this investigation a determination has been made of the least concentration of *Crotalus atrox* and of *Cobra* venom required to kill the animals within one hour. This, for each species, is called the minimal lethal concentration. The method used in determining this minimal lethal concentration is the same as that described in a previous publication (1930). The minimal lethal concentration of each venom for each of the species of Protozoa is shown in the accompanying table.

An inspection of the data shown in the table reveals the fact that the venoms at some concentration have a lethal effect on each of the species of Protozoa used. It is evident, however, that the Protozoa

Minimum Lethal Concentration of Crotalus atrox and Cobra Venoms for Fourteen Species of Protozoa

Species of Protozoa	Minimal Lethal Concentration	
	Cobra Venom, gm. per cc.	Crotalus atrox Venom, gm. per cc.
Amœba dubia	.000008	.001500
Blepharisma undulans	.000015	.000100
Chilomonas paramecium	.000010	.000300
Coleps hirtus	.000020	.000900
Colpidium campylum	.000006	.003000
Euglena gracilis	.000040	.000200
Euplotes patella	.000010	.000150
Oxytrichia fallax	.000040	.000150
Paramecium multinucleatum	.000010	.000050
Prorodon teres	.000025	.000300
Stentor polymorphus	.000200	.000100
Trachelius ovum	.000060	.000900
Urocentrum turbo	.000004	.000100
Vorticella sp.	.000100	.002000

exhibit a great variation in respect to the degree of the resistance of each species to the venoms. All species are more resistant to *Crotalus atrox* venom than to *Cobra* venom. Some species (*Urocentrum, Paramecium, Euplotes, Blepharisma*) are, in relation to other species, highly susceptible to both venoms. One species (*Vorticella*) has relatively great resistance to both. Some species (*Colpidium, Amœba, Chilomonas, Coleps* and *Prorodon*) are highly resistant to *Crotalus* venom but quite susceptible to the effects of *Cobra* venom. On the other hand, one species (*Stentor*) is, in relation to the other species, highly resistant to *Cobra* venom but susceptible to the action of *Crotalus* venom. The same is true to some extent with *Trachelius.*

Repeated titrations made in connection with previous studies have shown that the minimal lethal concentration for *Paramecium multinucleatum* of *Cobra* venom and of *Crotalus atrox* venom is respectively 0.0000016 gram and 0.00002 gram per cc., while in this study it is, respectively, 0.00001 gram and 0.00005 gram per cc. This indicates that the stock solution of *Cobra* venom used in this investigation was approximately one-sixth normal strength, while that for *Crotalus atrox* venom was two-fifths of its normal strength. Repeated tests were made with *Paramecia* to show that the stock solutions of the venoms used throughout this study did not deteriorate in strength during the time. The stock solution in each case consisted of 0.05 gram of dry venom dissolved in 4.5 cc. of distilled water to which 0.5 cc. of glycerine was added. The stock solutions were rendered neutral by the addition of Na_2HPO_4.

It may be concluded that, so far as this group of Protozoa is concerned, the poisonous effect of *Crotalus atrox* venom and of *Cobra* venom on Protozoa is general. There exists among the species, however, much variation in the resistance to the action of the two venoms, some species having relatively high resistance to the one and low to the other, while, in other species, the reverse relationship exists.

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A DETERMINATION OF THE TENSION AT THE SURFACE OF EGGS OF THE ANNELID, *CHÆTOPTERUS*

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By means of the microscope-centrifuge, recently described by Harvey and Loomis (1930), it is possible to observe, and to photograph, using highest dry objectives, living cells while subjected to centrifugal forces thousands of times gravity.[1] The cells are mounted on a special slide fixed over the objective, which rotates on the centrifuge at a distance of 11 cm. from the axis of rotation. By total reflecting prisms the image is brought to the axis of the centrifuge and then vertically upward where it is observed with a stationary ocular. The light, a condenser discharge in mercury vapor, is arranged for stroboscopic illumination, flashing on with each revolution of the centrifuge. The image is perfectly clear and steady at 4000 R.P.M.

Chætopterus eggs, as is well known from the work of F. R. Lillie (1909), pull apart into fragments under the influence of high centrifugal forces. The centrifuge-microscope allows us to observe clearly the series of changes in this process and to make exact measurements from which it is possible to calculate the tension at the surface of the egg. This process is illustrated for unfertilized *Chætopterus* eggs by the photographs of Fig. 1.

The first three rows show ten stages in the formation of a fragment or spherule containing oil, taken through the microscope-centrifuge while revolving about 4000 R.P.M., 5 seconds' exposure. It will be noted that No. 5 is a double exposure (by mistake) and shows the exact change in position which takes place in 1 minute. At one stage the protoplasm is drawn out into long filaments with the oil spherule at one end. In the pulling off of this oil spherule the appearance is that of a drop of molten glass slowly falling from a heated rod. When the oil finally breaks away, it moves slowly (as long as it can be observed in

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[1] The microscope-centrifuge was devised in collaboration with Mr. Alfred L. Loomis and constructed in his private laboratory at Tuxedo Park, New York. The author expresses his deep appreciation to Mr. Loomis for the hospitality of the laboratory. It is contemplated that the instrument will be placed on the market by the Bausch and Lomb Company. For description see Harvey and Loomis in *Science*, 1930, **72**: 42.

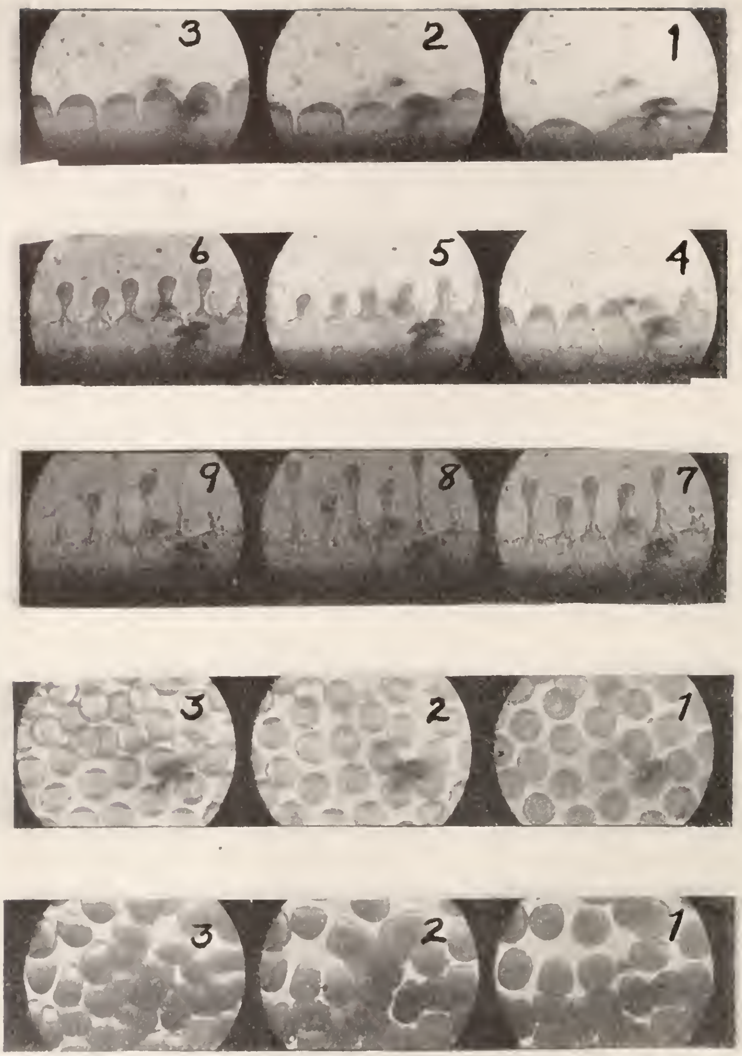

FIG. 1. Photographs of unfertilized eggs taken with a Leica camera through the microscope-centrifuge (radius 11 cm.), showing successive changes (examine from right to left) in stratification of granules and in shape of eggs. Magnification, 87 diameters. The direction of the centrifugal force is downward.

First three rows, *Chætopterus* exposed for 5 seconds at 35, 55, 95, 165, 205 and 265 (double exposure), 345, 375, 450, and 480 seconds after starting centrifuge. Speed of the microscope-centrifuge was 55 R.P.S. in the first row and 66 R.P.S. in the second and third rows. At the highest speed about 330 flashes of light occurred during the exposure.

Fourth row, *Cumingia* exposed 10 seconds at 35, 95 and 155 seconds after starting centrifuge. Speed 38 to 55 R.P.S. The eggs are separated by jelly.

Fifth row, *Arbacia* exposed 10 seconds at 45, 150, and 285 seconds after starting centrifuge. Speed 35 R.P.S. The eggs are partially separated by jelly.

the field of the microscope) to the surface of the sea water. Sometimes a clear fragment containing no yolk or oil globules will separate. These clear fragments are denser than sea water and sink; in fact, a clear fragment, one half containing oil globules, is heavier than sea water.

The pulling away of an egg spherule containing oil occupies about seven minutes at 1916 times gravity, and it will be noted that when an oil spherule breaks away the stalk does not round up immediately but remains for some time and only slowly rounds off. The stalk does not behave as if it were elastic like rubber, which would immediately recover when stretched, nor is it stiff, for one can observe these stalks waving back and forth in currents in the sea water during centrifuging. They appear like a very slow flowing plastic material such as tar.

The stalk is part of the original surface of the egg. Are we to regard that as having purely a surface tension at its boundary or a membrane of some sort with an elastic tension and a definite breaking strength, or should we think of the breaking strength of the stalk per cross sectional area? Perhaps the magnitude of this tension will allow us to decide. If we determine the buoyant force of the oil tending to draw apart the egg into fragments, we can equate this to a tension of the egg considered as acting only around the surface (surface tension or elastic tension of a membrane) or as acting over the whole cross sectional area of the stalk (internal friction of a plastic fluid), or a combination of the two.

Fortunately, it is easy to gain a rough idea of the buoyant force, which tends to pull apart the egg, by the following considerations. A maximum value for this force will be obtained if we consider oil rising through egg fluid rather than egg spherule rising through sea water. Let us assume the density of the oil is 0.915, the same as that of hen's egg yolk oil. By measuring the diameter of the oil spherule ($34\,\mu$) pulled away from the egg, we find that the volume of the oil is 75 per cent[2] of 2.05×10^{-8} cc. or 1.54×10^{-8} cc., and its mass is 1.41×10^{-8} grams. The density of the medium can be taken as 1.044, the average of Heilbrunn's (1926) values for *Arbacia* and *Cumingia* eggs, and slightly greater than sea water (1.0238) at Woods Hole at 20° C. Therefore, the buoyant force of the oil pulling inward toward the axis of the centrifuge will be $(1.044 - 0.915)/0.915 \times 1.41 \times 10^{-8} = 0.2 \times 10^{-8}$ grams $\times\ 980 \times$ centrifugal force $(1916 \times g) = 3.75 \times 10^{-3}$ dynes. Since the yolk rests against the bottom of the slide, this will be the only force tending to pull the egg apart, and it will be counteracted by the surface tension or elastic tension (T) of the egg, acting around the circumference (πD) of the stalk of diameter (D) connecting oil spherule and egg at the moment the stalk breaks.[3] Hence $\pi DT = 3.75 \times 10^{-3}$ dynes.

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[2] Since the oil granules are spheres and spheres packed in a volume occupy only 75 per cent of that volume.

The diameter of the stalk is found to be 9 μ by measurement. Solving, $T = 1.32$ dynes per cm. This is a maximum value, as it is likely the oil might have been pulled away with somewhat lower centrifugal forces, although it takes 7 minutes to pull apart at 1916 × g. One dyne per cm. cannot be very far from the truth, perhaps 25 per cent error at most. If we are to regard this as a surface tension, it is very low, but might be compared to that of a water—isobutyl alcohol interface whose T is 1.76 dynes per cm. at 20° C. On the other hand, the value seems entirely too low for an elastic membrane tension.

If we regard the stalk of *Chætopterus* eggs as made up of homogeneous material, its breaking strength, S, would be $(\pi/4)D^2S = 3.75 \times 10^{-3}$ dynes or $S = 5900$ dynes per cm.^{2}, also an extremely small value. The breaking strength of rubber is about 100 million dynes per cm.^{2}

The long cylindrical stalk of centrifuged *Chætopterus* eggs would be a very unstable figure according to classical surface tension interpretation. Plateau showed that a cylinder breaks into drops when its length equals its circumference. This is a geometrical relation, independent of the value of the surface tension, provided the fluids have a low viscosity. In the derivation of surface tension formulas by the vibrating drop and vibrating jet methods the viscosity of the fluid is neglected for simplification. With very high viscosities the attainment of equilibrium conditions might take so long a time as to make thin cylinders stable figures for all practical purposes. With plastic fluids surface tension might be too small to overcome the internal friction.

The value of 1.3 dynes per cm. does not allow us to decide definitely whether the boundary of the egg exerts a surface tension or an elastic tension, but it does show that there can be no very firm "pellicle" around these eggs. The observed behavior of the stalk makes it quite certain that we are dealing with a very plastic material. If the tension were greater than fifty dynes per cm., a maximum value for liquid-water interfaces, we might say quite definitely that it was elastic membrane and not surface tension. Such may be the case in eggs like *Arbacia* and *Cumingia* which are not torn apart by much higher centrifugal forces, and show only a slight tendency to lengthen, as illustrated in rows 4 and 5 of Fig. 1.

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[3] I am deeply indebted to Dr. Charles Zahn of the Physics Department, Princeton University, for suggestions regarding the interpretation of some of these surface tension phenomena.

SUMMARY

A method is described, using the microscope-centrifuge, for calculating the tension at the surface of *Chætopterus* eggs, which gives an approximate *maximum* value of 1.3 dynes per cm.

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THE EFFECT OF CERTAIN NARCOTICS (URETHANES) ON PERMEABILITY OF LIVING CELLS TO WATER

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The experiments on narcotics reported in this paper were carried out with the purpose of gaining further information on the factors which regulate or influence permeability of the living cell to water. Former studies on sea urchin eggs have shown that cell permeability to water is not constant, but varies with a number of factors, an important one being the chemical composition of the medium (McCutcheon and Lucké, 1928). Thus permeability is regulated, at least in part, by the sign and the number of charges on the ions of the medium, in the sense that anions increase and cations decrease permeability to water; the effects are the greater the higher the valence of the ions (Lucké and McCutcheon, 1929). In the presence of cations of two or more valences permeability is of very low magnitude. For example, in hypotonic sea water cells, such as unfertilized eggs of the sea urchin, have a numerical value of permeability of approximately 0.05 at 15° C., *i.e.*, 0.05 cubic micron of water pass through each square micron of cell surface per minute, under the driving force of one atmosphere of osmotic pressure.[1] This surprisingly low degree of permeability is presumably due to the high concentration of calcium and magnesium in sea water, for the same low value of permeability is obtained when cells are placed in a non-electrolyte medium, of like osmotic pressure, containing as little as 0.0001 molar $CaCl_2$ or $MgCl_2$. But all attempts to further decrease permeability have so far been unsuccessful. However, several writers have reported that narcotics decrease permeability to water (Winterstein, 1916; Lillie, 1918; and Anselmino, 1928). The question therefore arose whether narcotics in the presence of sea water, or narcotics in any solution containing cations of two or more valences, would lower permeability beyond the value obtained in sea water alone.

METHOD

A satisfactory method of studying quantitatively the effect of various factors on permeability to water is to place a suitable cell in a hypotonic medium, thus causing water to enter the cell under the driving force of osmotic pressure. The spherical unfertilized egg of the sea urchin, *Arbacia punctulata*, is an excellent natural osmometer (McCutcheon and Lucké, 1926; McCutcheon, Lucké and Hartline, 1931 *a* and *b*). When placed in a hypotonic solution it swells relatively slowly, thus permitting accurate measurement of its diameter, from which volume and surface area can be calculated; it has a high degree of semipermeability, retains its spherical shape during change of volume, and allows ready determination of injury which may have taken place during the experiment.

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[1] By permeability to water is understood the quantity of water passing through unit area of cell surface in unit time under unit pressure.

The narcotics selected for this study are certain urethanes and carbamates.[2] These compounds have the advantage of being non-injurious over a considerable range of concentration; they are non-volatile, penetrate almost instantaneously, and their narcotic effect is easily demonstrable with the material used.[3]

In the first series of experiments the narcotics were dissolved in ordinary (100 per cent) sea water. Unfertilized eggs of *Arbacia* were exposed to these solutions for from 5 to 10 minutes. The cells were then transferred to hypotonic sea water (usually 40 per cent sea water) with the narcotic to be tested in the same concentration as in the isotonic solution. The course of inflow of water at constant temperature (15 ± 0.5° C.) was then observed by measuring the diameter of three cells at minute intervals with a filar micrometer for six successive minutes.[4] The mean volume of the cells was plotted against time and a smooth curve drawn through the points. The rate of passage of water is given by the rate of change of volume, dV/dt, and is obtained from the slope of the curve at a given time, t. The numerical value of permeability to water is calculated from the equation:

$$\text{Permeability} = \frac{dV}{dt} / S(P - P_{\text{ex}}),$$

where S is the surface area of the cell and $(P - P_{\text{ex}})$ the difference in pressure between the interior of the cell at time t and the medium.[5]

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[2] For the sake of brevity the compounds will be referred to in this paper as urethanes. The propyl urethanes were not commercially obtainable, and were kindly supplied by Dr. Ralph Major, then of Princeton University.

[3] The narcotic effect of these compounds will be dealt with by Dr. E. B. Harvey in a forthcoming paper.

[4] The technic of measuring volume changes in *Arbacia* eggs by means of a filar micrometer eye-piece has been previously described (McCutcheon and Lucké, 1926).

[5] For details of calculation see McCutcheon and Lucké, 1928, 1929, and Lucké, Hartline and McCutcheon, 1931*b*.

EFFECT OF URETHANES WHEN DISSOLVED IN SEA WATER

Before determining the effect of these narcotics on permeability a preliminary question needed to be answered: Do these compounds affect the volume of cells in equilibrium with either an isotonic or hypotonic medium? If they should not enter practically instantaneously the volume of the cells would decrease when transferred to isotonic sea water containing a considerable concentration of the narcotic (*e.g.*, 0.2 m. ethyl urethane). On the other hand, if these compounds do enter rapidly, but cause severe injury or death, the volume of the cell in equilibrium with a hypotonic solution might be either too small (from escape of dissolved substances) (Lucké and McCutcheon, 1930) or too large (from splitting of substances in the interior of the cell) (Lucké and McCutcheon, 1926). Now, in order to use the simple equation for permeability given above it is necessary that the equilibrium volume of the cells in the solution to be tested should be the same as the volume at equilibrium of control (unnarcotized) cells in both isotonic and hypotonic concentrations of their natural medium, sea water. This question was answered as follows:

Different lots of cells from the same animal were placed in sea water in which had been dissolved a narcotic in the concentrations shown in Table I. After 5 to 10 minutes' exposure a number of cells were measured and then transferred to a hypotonic solution containing the same concentration of the urethanes. The volume at equilibrium was

TABLE I

Effect of Urethanes on Cell Volume at Equilibrium

The concentrations shown are the highest used in the experiments. V_0 is the mean volume of 25 cells measured after from 5 to 10 minutes' exposure to the narcotic solution in isotonic (100 per cent) sea water, V_e is the mean volume of 25 cells in equilibrium with a given hypotonic solution in which were dissolved the different urethanes (measured 4 hours after transfer). It is seen that the volume of cells in the narcotic solutions corresponds closely with the volume of control cells in the same concentration of sea water. The figures must be multiplied by 100 to obtain volumes in cubic micra.

Solution	V_0	V_e
First sea water control	1908	3255
Second sea water control	1882	3230
Ethyl urethane 0.2 m	1933	3182
n-propyl urethane 0.1 m	1920	3230
i-propyl urethane 0.1 m	1876	3182
n-butyl carbamate 0.05 m	1884	3275
Phenyl urethane 0.002 m	1924	3273

determined after 4 hours' exposure at 22° C. Table I shows the result of a representative experiment. It is seen that there is no change in volume of the narcotized cells in isotonic sea water, and the final equilibrium attained in hypotonic solutions corresponds to that of the control (unnarcotized) cells.[6] Other experiments gave similar results.

It was, therefore, possible to study the effect of these narcotics on cellular permeability to water by the method outlined above. In Table II are given three experiments, representative of a larger number. The table shows that none of the urethanes over a considerable range of definitely narcotic concentration caused significant change in permeability from the controls, excepting that increase in permeability occurred when the cells became injured during the experiment.[7]

These and similar experiments lead to the conclusion that urethanes in narcotic concentration when dissolved in sea water do not decrease permeability of the living cell to water.

EFFECT OF URETHANES WHEN DISSOLVED IN A NON-ELECTROLYTE MEDIUM

The result obtained with narcotics when dissolved in sea water, *i.e.*, failure to decrease cell permeability to water, may possibly be explained on the grounds that at a given temperature permeability can be reduced only to a certain value, and that this value is the one normally obtained in sea water. Since, then, the presence of the bivalent cations of sea water might, perhaps, mask the action of narcotics, the experiments were repeated in hypotonic dextrose solution.

The experiments were carried out as follows: Unfertilized eggs of *Arbacia* were washed in 0.95 molal solution of dextrose, isotonic with sea water, to eliminate electrolytes from the medium. The cells were then caused to swell in 0.38 molal dextrose solution, isotonic with 40 per cent sea water. Other eggs from the same animal were washed in isotonic dextrose solutions containing in the one case urethane, in the other calcium chloride; they were then measured during the course of swelling in hypotonic dextrose solution to which had been added ure-

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[6] It should be pointed out that *prolonged* exposure to urethane solutions may change the shape of cells. This is especially the case in the more concentrated solutions of ethyl and propyl urethane, in which the normally spherical cells may become transformed to bizarre amoeboid forms. No such changes occurred when cells were exposed to less concentrated solutions, for a shorter length of time and at a lower temperature. In the experiments here reported, cells retained their normal shape.

[7] The narcotics, in the experiments here reported, were used in three different concentrations, of which the lowest was still definitely narcotizing and the highest not toxic under the conditions of the experiment. Still higher concentrations proved injurious and increased permeability.

The narcotizing effect was kindly determined by Dr. E. B. Harvey on the basis of cleavage experiments.

TABLE II

Urethanes in Sea Water

In these three experiments the narcotics in the molar concentrations shown are dissolved in 40 per cent sea water (40 parts of sea water and 60 parts of distilled water). In the last column of the table is given the value of permeability, which is the number of cubic micra of water entering the cell per minute, per square micron of surface, per atmosphere of pressure. The temperature was 15 ± 0.5° C.

It is seen that the various urethanes do not decrease permeability beyond the value obtained in sea water alone. The increased values of permeability (indicated by an asterisk) were obtained in injured cells, *i.e.*, in cells which failed to cleave when returned to ordinary sea water and inseminated at the conclusion of the experiment.[8]

Compound	Concentration	Permeability
Ethyl urethane	0.2	0.055
" "	0.1	0.057
" "	0.05	0.056
First sea water control		0.054
Second sea water control		0.054
n-propyl urethane	0.1	0.096*
n-propyl urethane	0.05	0.052
n-propyl urethane	0.025	0.056
i-propyl urethane	0.1	0.057
i-propyl urethane	0.05	0.051
i-propyl urethane	0.025	0.055
First sea water control		0.058
Second sea water control		0.053
n-butyl carbamate	0.05	0.089*
n-butyl carbamate	0.025	0.061
n-butyl carbamate	0.0125	0.063
i-amyl carbamate	0.01	0.059
i-amyl carbamate	0.005	0.061
phenyl urethane	0.005	0.060
phenyl urethane	0.0025	0.057
phenyl urethane	0.00125	0.056
First sea water control		0.059
Second sea water control		0.069

[8] It has previously been shown (Lucké and McCutcheon, 1930) that injury induced by high temperature and by anisotonic solution causes an increase in cellular permeability to water. From the experiments given in the table as well as from similar experiments, it is evident that injury induced by toxic concentrations of narcotics also increases permeability to water. Higher concentration of narcotics than those employed produced injury, and hence increased permeability.

thane or calcium, in the same concentration as was present in the isotonic solution of dextrose. Permeability was therefore determined, in each experiment, in pure dextrose solution, in dextrose solution containing a narcotic, and in dextrose containing calcium.

The results of three such experiments are shown in Table III. It is seen that the values of permeability in pure dextrose solution are about twice as great as in calcium-dextrose solution, while permeability values of the narcotized cells lie about midway. A number of similar experiments gave the same results. In every case exposure to the narcotic caused a definite decrease in permeability, which was never of the magnitude of the decrease effected by calcium. The conclusion may be drawn that narcotics tend to decrease cell permeability to water but that their effect may be masked by the presence of cations in the medium.

TABLE III

Urethanes in Dextrose

The narcotics in the molar concentrations shown are dissolved in 0.38 molal solution of dextrose. In the top row is given the permeability of cells in pure dextrose solution and in the bottom row the permeability in dextrose solution containing 0.01 m. $CaCl_2$. Each permeability value in this experiment is based on measurements of ten cells. The temperature was 12° ± 0.5° C.

It is seen that the narcotics cause a definite decrease in permeability which, however, is not as great as the decrease effected by calcium.

Solution	Permeability		
Dextrose 0.38 m	0.096	0.097	0.092
Dextrose 0.38 + n-butyl carbamate 0.025 m	0.062		
Dextrose 0.38 + i-amyl carbamate 0.01 m		0.085	
Dextrose 0.38 + phenyl urethane 0.0025 m			0.070
Dextrose 0.38 + $CaCl_2$ 0.01 m	0.041	0.047	0.041

DISCUSSION

Most studies of the effect of narcotics on cell permeability have been concerned only with permeability to various substances in solution.[9] The most important investigations on permeability to water, as influenced by narcotics, are those of Winterstein (1916), Lillie (1918), Heilbrunn (1925) and Anselmino (1928).

Winterstein in his first group of experiments used sartorius muscle of frogs, employing the usual method of weighing. He found that in hypotonic solution of sodium chloride containing alcohol in narcotic concentration the weight increase of muscle was less than in the same hypotonic solution containing no narcotic. In later experiments Winterstein constructed artificial "cells"; glass cylinders were covered with the thin abdominal muscle of female frogs. It was found that, in agreement with the experiments on sartorius muscle, four different narcotics effected a marked decrease of the water intake by the "cell." The effects were reversible.

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[9] The literature on this subject is reviewed by Gellhorn, E., Das Permeabilitätsproblem, Berlin, 1929; Winterstein, H., Die Narkose, Berlin 1926; Lillie, R. S., Protoplasmic Action and Nervous Action, Chicago, 1924; Bayliss, W. M., Principles of General Physiology, 4th edition, London, 1924; Osterhout, W. J. V., Injury, Recovery, and Death in Relation to Conductivity and Permeability, Philadelphia and London, 1922; Jacobs, M. H., in Cowdry, E. V., General Cytology, Chicago, 1924; Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926; von Tschermak, A., Allgemeine Physiologie, I, Berlin, 1924.

Lillie investigated the effect of narcotics on permeability to water of fertilized eggs of *Arbacia*. He had previously shown that in the *Arbacia* egg fertilization is followed by an approximately four-fold increase in permeability to water. This increase of permeability was found to be inhibited by various organic anesthetics. In all cases eggs which were caused to shrink, two or three minutes after insemination, in solutions of these compounds in sea water of the appropriate conconcentrations, remained in the condition of low permeability characteristic of the unfertilized egg. This effect of narcotics was readily reversible.

Heilbrunn repeated Winterstein's experiments, studying the increase in weight of frog's gastrocnemius muscle in distilled water, and in distilled water to which two per cent by volume of ether had been added. He found that water entered etherized muscle somewhat less rapidly than normal muscle. In further experiments Heilbrunn investigated the rapidity of swelling of unfertilized eggs of *Arbacia* in hypotonic sea water containing one or two per cent of ether. His curves show that the cells swelled even more readily in the presence of ether than in its absence. From these experiments Heilbrunn concluded that ether does not lower the permeability of sea urchin eggs to water.[10]

Very recently, Anselmino investigated the effect of various narcotics on permeability to water of dried collodion and of copper ferrocyanide membranes, and found, in agreement with the work of Winterstein on living membrane, a marked decrease of permeability to water.

From the experiments summarized above (excepting Heilbrunn's experiment on *Arbacia* eggs) the conclusion was drawn that narcotics decrease permeability to water. It would appear, however, that the chemical composition of the medium is a factor of importance when investigating the action of narcotics on cell permeability. The experiments reported in the present paper indicate that narcotics, at least in the case of the unfertilized egg of *Arbacia*, do not lower permeability to water when they are caused to act in sea water, or in a medium containing calcium.

[10] It is not improbable that the more rapid swelling of the narcotized cells in Heilbrunn's experiments is due to injury.

SUMMARY

1. The effect of narcotics (urethanes and carbamates) on cell permeability to water was studied by measuring the rate of swelling of unfertilized eggs of the sea urchin, *Arbacia punctulata*, in hypotonic sea water and in hypotonic dextrose solution.

2. Narcotics in the presence of sea water do not decrease permeability to water beyond the value normally found in sea water.

3. But narcotics have a tendency to reduce permeability to water, being, however, less effective in this respect than are bivalent cations. This tendency to decrease permeability is demonstrated when narcotics are used in solutions free from bivalent cations, *i.e.*, in hypotonic solutions of dextrose.

4. The effect of narcotics on permeability to water depends on the chemical composition of the medium in which the narcotizing compound is dissolved.

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THE CHANGES DURING DESICCATION AND REHYDRATION IN THE BODY AND ORGANS OF THE LEOPARD FROG (*RANA PIPIENS*)

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On account of the fundamental importance of water in the living organism, it is of interest to know how much water the body can lose from its storage depots and yet survive. The further question arises as to whether there is, after recovery from desiccation, a normal redistribution of the water in the body as shown by the water content of the various organs. Dehydration is difficult to produce in the mammalian organism, but is easily accomplished in the frog. This animal was therefore used in the present experiments.

MATERIAL AND METHODS

Forty common leopard frogs (*Rana pipiens*) were used in the experiments. They were all males of similar weight and obtained from the same location in southern Minnesota. They were caught in October, and the experiments ran from October through February. The frogs were kept in large concrete tanks through which ran fresh, cold tap water at a temperature ranging from 8° to 11° C. The water was kept at a depth of six inches. Bricks were put into the tanks in such a manner that when the frogs rested on them, only their heads would be exposed. The frogs had reached a constant body weight before being used.

The forty animals were divided into four groups of ten frogs each. These were designated as control group A (controls for normal fresh organ weights); control group B (controls for normal total water content); test group C (frogs desiccated for organ weights), and test group D (rehydrated for organ weights). The frogs in control group A were anesthetized with ether and autopsied at once. Their fresh and dry organ weights were determined. The control group B were killed by the ether and their fresh and dry weights were determined for the body as a whole. The test group C were desiccated so as to lose forty per cent or more of their total body weight, then anesthetized and autopsied to obtain the fresh and dry organ weights. The test group D were desiccated to the same extent as group C, and then allowed to recover for ten days in water at room temperature of 25° C. (range 20–28° C.). They were then anesthetized and autopsied to secure the fresh and dry organ weights. The body weights of test groups C and D were taken at frequent intervals (see Fig. 1) to determine the rate of dehydration, and, in the case of group D, also the rate of rehydration.

The frogs were desiccated by exposing them to evaporation, each in a cylindrical glass jar 8 inches in height and width. These jars were covered by a metal screen with three meshes to the inch. They were placed in a room, the temperature of which was kept fairly constant by means of a thermostat. The average temperature was 25° C. (range 23–26° C.). The average relative humidity was 25 (range 20–29), measured with a Tycos hygrometer.

Before weighing in all groups, the excess of moisture was removed from the frogs by carefully blotting with paper towels. No allowance was made for the contents of the urinary bladder, as the frogs usually voided urine during the manipulation incident to drying them.

In all cases, ether was used to anesthetize the frogs. With the exception of control group B, the (anesthetized) animals were killed by cutting open the tip of the cardiac ventricle and suspending the animals by the head and feet so that all the blood possible might drain out into a bottle. The weight and the water content of the escaped blood were determined.

The procedure at autopsy was as follows: The head was severed from the body at the atlanto-occipital articulation. The limbs were separated at the hip and shoulder joints, respectively. All parts were placed in a moist chamber until dissected. The dissection was made upon dampened blotting paper. The lungs were removed at the junction with the trachea, and were opened to remove any parasitic flukes found there. The heart was removed by cutting the large veins at their termination, and severing the aorta at its origin from the bulbus aortae. The chambers of the heart were opened and their contents (if any) removed. The contents of the stomach and intestines were removed before weighing these organs. The ano-cloacal junction was taken as the lower border of the intestines. The suprarenal glands were not removed from the kidneys. The liver was removed and weighed together with the gall bladder. The bile in all cases was allowed to drain out before the weighing. The tongue was separated by cutting the muscles at their attachment to the hyoid bone. When the integument was taken, the subjacent lymph was merely allowed to drain off. The term "remainder" includes the various items such as large blood vessels, nerves, connective tissue and other structures not included under the other headings. The remainder does *not* include the water in the subcutaneous lymph spaces.

All organs were weighed on a Wilkens-Anderson analytical balance in previously weighed weighing bottles with ground glass stoppers. The organs, and likewise the entire frogs of control group B, were dried in a Thelco electric oven at about 95° C. until constant weights were obtained.

RESULTS

General Observations

The frogs of all groups were healthy and normal in all respects. The average and range of initial body weights in grams were as follows: control group A, 43.59 (35.0 – 53.2); control group B, 40.64 (34.5 – 41.6); test group C, 43.57 (37.0 – 53.1); test group D, 44.80 (42.7 – 49.0). It will be noted that in average body weight groups A and C were nearly identical, and group D differed from these by about only two per cent. In the calculations, it is assumed that the initial organ weights were equal in these three groups.

It required 28.6 hours, on the average, to desiccate the test group C to an average weight of 24.98 grams, with loss of 42.7 per cent of their initial body weight, or 51.9 per cent of their total water content (Fig. 1).[1] These frogs at first were quite active, but after a few hours of desiccation they remained motionless, apparently almost lifeless. The skin became very dry, and the animals appeared emaciated. The frogs maintained the sitting position, and the joints of the limbs became stiff. In addition to the ten surviving frogs in this group, four others died during the test and were excluded from the experiment.

A period of 31.8 hours was required to desiccate the test group D to an average weight of 25.40 grams, with loss of 43.3 per cent of their average initial body weight or 52.7 per cent of their total water content.[1] Up to this stage, the general appearances and reactions were the same as in the test group C. Upon being put back into water at room temperature (25° C.) for rehydration, the test group D at first remained inactive. After two or three hours, they gradually began to move about, and soon became normal to all outward appearances. After 26.2 hours these frogs had returned to a nearly constant body weight, averaging 36.74 grams (Fig. 1). This weight they maintained, with slight fluctuations, for ten days before being autopsied. This nearly constant body weight averaged about 8.1 grams below the original body weight. Figure 1 shows the general weight curve followed by this group during desiccation and rehydration. In addition to the ten surviving frogs in this group, five others died during the test and were excluded from the experiment. These five frogs died during the dehydration part of the experiment. No deaths occurred during rehydration.

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[1] These percentages for the estimated losses of water content are slightly too high, since they include (as shown later) a loss of about 0.51 gram in solids during the experiment. The corrected loss of total water content is 50.5 per cent for group C and 51.3 per cent for group D.

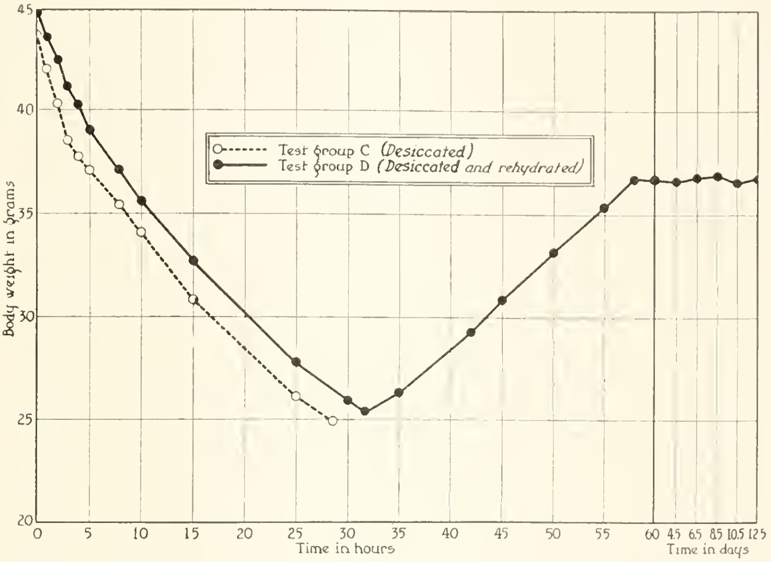

FIG. 1. Curves of average weight during desiccation and rehydration. Test group C was desiccated 28.6 hours, decreasing in average body weight from 43.6 grams to 25.0 grams. This corresponds to a loss of about 43 per cent in body weight. Test group D was similarly desiccated 31.8 hours, decreasing from 44.8 grams to 25.4 grams, with loss of about 43 per cent in body weight. This group was then replaced in water and increased to 36.7 grams in 26.2 hours (end of 58th hour of experiment). Thereafter the weight remained nearly stationary during a period of 10 days. Failure to reach the original weight is ascribed to the increase in room temperature.

Observations on Individual Organs and Parts

Observations at Autopsy.—At autopsy the control group A appeared normal in every respect with the exception of an occasional lung fluke. The test groups C and D also occasionally contained one to three flukes. In the desiccated test group C the blood was so thick that it would barely run into the container. There seemed to be practically no water in the tissue spaces and lymph sacs. The intestinal contents were very slight. Most of the organs appeared very dry.

The rehydrated test group D at autopsy appeared quite normal with the exception that the epidermis was shedding in large pieces quite generally over the body. This process resembled the normal ecdysis, although in this case it was caused by the desiccation. The amount of water in the tissue spaces and lymph sacs appeared somewhat below that in the normal control group A. The blood seemed normal in color and consistency.

Fresh Weights of Organs.—(Tables I and II.)—The average fresh organ weights of control group A were taken as norms, and the average fresh organ weights of test groups C and D were compared with these.

TABLE I

Average Fresh Organ Weights in Grams

	Control group A (normal)	Test group C (desiccated)	Test group D (rehydrated)
Eyeballs	0.3980	0.3958	0.3987
Lungs	0.2146	0.1992	0.2134
Skeleton	5.2250	5.0073	5.1246
Brain	0.1013	0.0934	0.1093
Kidneys	0.1381	0.1151	0.1442
Heart	0.1112	0.0922	0.1057
Spinal cord	0.0636	0.0498	0.0591
Testes	0.0424	0.0314	0.0456
Stomach-intestines	0.6182	0.4326	0.5812
Muscles	16.5533	11.4997	16.0331
Skin	5.7809	3.3976	4.7196
Tongue	0.3993	0.1892	0.3800
Spleen	0.0381	0.0170	0.0296
Liver	1.6586	0.7019	1.0137
Blood	1.4274	0.2739	1.3468
Fat bodies	0.0950	0.0251	0.0463
Remainder	0.7194	0.4448	0.7808
Total	33.5844	22.9660	31.1317

As shown by Tables I and II, in the test group C, the eyeballs and lungs remained nearly unchanged in weight, and the skeleton and brain lost less than ten per cent through desiccation. The kidneys, heart, spinal cord, testes, stomach-intestines and muscles form a group losing moderately (16–30 per cent), but relatively less than the body as a whole. Finally the skin, tongue, spleen, liver and (escaped) blood lost 43–81 per cent, which exceeds the relative loss of the body as a whole.

In the rehydrated test group D, all the fresh organs, with the exception of the skin, spleen and liver, had regained practically normal weight (within 8 per cent). The skin remained about eighteen per cent below the norm, the spleen 22 per cent below, and the liver nearly 39 per cent below. The significance of these changes will be considered later.

TABLE II

Average Percentage Difference in Fresh Organ Weights of Test Groups C and D, Compared with Control Group A

	Test group C (desiccated)	Test group D (rehydrated)
1. *Organs with slight loss during desiccation*		
Eyeballs	−0.6	±0.0
Lungs	−0.6	−7.2
Skeleton	−4.2	−1.9
Brain	−7.8	+7.9
2. *Organs with moderate loss during desiccation*		
Kidneys	−16.7	+4.4
Heart	−17.1	−5.0
Spinal cord	−21.7	−7.1
Testes	−26.0	+7.6
Stomach-intestines	−30.0	−6.0
Muscles	−30.5	−3.1
3. *Organs with great loss during desiccation*		
Skin	−42.8	−18.4
Tongue	−52.6	−4.8
Spleen	−55.4	−22.3
Liver	−57.7	−38.9
Blood	−80.8	−5.6

Water Content.—(Tables III and IV.)—The average percentage water content found in the whole body of control group B was 82.14 per cent, ranging from 80.21 to 84.28 per cent. This average is taken as the norm for the body as a whole.

The average percentages of water found in the organs of control group A, and of test groups C and D, are given in Table III. For the normal control group A, the organs are arranged in order of increasing relative water content, from the skeleton (58.13) to the escaped blood (88.97 per cent). The muscles in this group contain almost exactly the same percentage of water as the average for the whole body. The skeleton, liver, spinal cord, skin and spleen contain relatively less, and the testes, "remainder," brain, heart, kidneys, stomach-intestines, tongue, lungs, eyeballs and blood contain relatively more water than the body as a whole.

In the desiccated test group C, the organs listed (Table III) have all lost part of their water and hence appear lower in relative water content than the corresponding organs of control group A. Assuming that all the loss of weight in this desiccated group C (Table I) is due to loss of water, we may estimate the theoretical water content of the organs in group C. This theoretical water content should agree fairly with that actually found in the desiccated test group C (Table III). The calculations of the theoretical water content were made in this way for every organ, but the figures in detail are omitted for economy of space. The muscles on this basis show the closest agreement with a discrepancy of only 0.2 per cent.[2] Agreement (within 3.5 per cent) is also found in the case of all the other organs except the liver, spleen, tongue, lungs and skin. The percentage of water in the liver of the desiccated group averages 28.7 per cent greater than that to be expected from its loss of weight. The corresponding percentages in the spleen, tongue, lungs and skin are, respectively, 17.9, 6.8, 4.5 and 4.0 per cent greater than those theoretically calculated from the change in organ weight. The significance of these discrepancies will be discussed later.

TABLE III

Average Percentage of Water in Organs

	Control group A (normal)	Test group C (desiccated)	Test group D (rehydrated)
Skeleton	58.13	57.94	60.52
Liver	76.08	72.10	76.41
Spinal cord	80.75	76.73	81.67
Skin	81.05	70.84	80.15
Spleen	82.13	77.84	81.39
Muscles	82.15	74.52	82.17
Testes	82.57	75.09	81.40
Remainder	83.12	77.19	81.85
Brain	83.90	81.28	85.09
Heart	84.07	82.24	87.17
Kidneys	84.42	78.09	84.06
Stomach-intestines	85.00	75.21	83.36
Tongue	86.13	76.83	87.87
Lungs	87.11	82.52	86.15
Eyeballs	87.83	85.38	88.16
Blood	88.97	77.33	88.79

The absolute amount of water given up by each of the various organs or parts in test group C is shown in Table IV. The amounts are estimated from the differences in relative water content between groups A and C (Table III), assuming that the initial organ weights were equal to those in control group A. The estimate for the lymph spaces is explained later. The chief storage depots of water in the frog's body are apparently the lymph spaces, yielding 8.51 grams (or 45.8 per cent of the total 18.5776 grams of water lost by the body during desiccation); and the muscles, yielding 5.03 grams (or 27.1 per cent). Somewhat smaller contributions are made by the skin (2.28 grams or 12.3 per cent), and the blood (1.06 grams or 5.7 per cent). All the other organs together give up only 1.70 grams, or 9.2 per cent.

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[2] The method of calculation is as follows: As shown in Table III, every 100 grams of fresh muscle in the normal control group A contains 82.15 grams of water and (accordingly) 17.85 grams of solids. Table II shows that in the desiccated test group C each 100 grams of muscle had lost 30.5 grams in weight, which is assumed to be due to loss of water. Subtracting 30.5 from 82.15 gives 51.65 grams of water remaining in the 69.50 grams of muscle in the desiccated frog. This gives a theoretical water content of 74.32 per cent. The actual water content, as found in the oven-dried muscle of the desiccated test group C (Table III), was 74.52 per cent, which differs from the theoretical value by only 0.2 per cent.

TABLE IV

Organs and Parts of Test Group C, arranged according to the Absolute Amount of Water in Grams Lost During Desiccation. Estimated on basis of change in percentage of water content shown in Table III and normal fresh organ weights in Table I.

Lymph spaces	8.5100	Fat bodies	0.0294
Muscles	5.0289	Lungs	0.0225
Skin	2.2794	Kidneys	0.0210
Blood	1.0582	Spleen	0.0181
Liver	0.7559	Heart	0.0177
Remainder	0.2547	Eyeballs	0.0134
Stomach-intestines	0.2001	Spinal cord	0.0132
Tongue	0.1985	Testes	0.0112
Skeleton	0.1363	Brain	0.0091
		Total	18.5776

In the frogs of the rehydrated test group D, the organs in general have regained nearly normal water content (Table III). All the organs average within 2 per cent of the normal water content found in control group A, excepting the skeleton, which is about 2.4 per cent above normal, and the heart, which averages 3.1 per cent above.

To determine the amount of water in the lymph spaces of control group A, the total average fresh organ weight (33.58 grams, Table I) was subtracted from the average original body weight (43.59 grams). This gives a difference of 10.01 grams, due chiefly to the contents of the lymph spaces. During desiccation, the test group C frogs lost 18.59 grams in average body weight. The organs of this group (excluding the lymph spaces) apparently gave up 10.07 grams of water, as shown by the estimates in Table IV. The remainder of the loss (8.52 grams) was assumed to come from the lymph spaces, although a small part of this loss is in solids, as will be shown later. It is assumed that the lymph spaces of test groups C and D originally contained as much water as the lymph spaces of control group A (10.01 grams). Therefore, at the time of autopsy the lymph spaces of test group C should theoretically contain 1.49 grams (10.01 minus 8.52 grams). If we subtract the average fresh organ weight of this group (22.97 grams, Table I) from their average final body weight (24.98 grams), we find that the actual amount of water in the lymph spaces was 2.01 grams. The apparent loss of solids in the various organs can be calculated by comparing the theoretical losses of water (Table IV) with the decreases in fresh weight (Table I). All the organs excepting the stomach-intestines, lungs, eyeballs and testes show variable (usually small) losses in weight beyond those due to apparent decrease in water content. The net decrease amounts to about 0.58 gram, or apparently slightly more than sufficient to account for the difference between the theoretical and the actual amount of water in the lymph spaces (0.52 gram).

The total theoretical amount of water in the body, as a whole, in the rehydrated test group D at time of autopsy was 30.18 grams. This is obtained from the average final body weight (36.74 grams) multiplied by the normal percentage of water in the whole body (82.14 per cent). If we subtract the total water actually found in the organs (24.46 grams) from the total (theoretical) water content of the body (30.18 grams), we get the theoretical amount of water in the lymph spaces (5.72 grams) of the rehydrated test group D. We can check this by subtracting the average fresh organ weight of test group D (31.13 grams, Table I) from the average final body weight for this group (36.74 grams). This gives 5.61 grams as the amount of water found in the lymph spaces, which is very close to the theoretical expectation.

In general, therefore, it appears that the lymph spaces of the desiccated test group C had given up approximately 80 per cent of their initial water content, while the lymph spaces of the rehydrated test group D had not fully refilled, but were still about 44 per cent below their estimated original water content. The explanation for this difference will be discussed later.

Discussion

In general, it must be remembered that the number of frogs (10) in each of the four groups is relatively small. The results may therefore be influenced somewhat by individual variations, so that in some cases the conclusions are to be regarded as tentative rather than final.

General Observations

The average loss of weight (about 43 per cent) sustained by desiccation in our test groups C and D may be compared with the results of other investigators. Chossat (1843) noted a loss of 35 per cent in the body weight of frogs subjected to evaporation. Similar observations were made by Durig (1901). Hall (1922) subjected various species of animals to exsiccation, and found that frogs survived a desiccation period of 33 hours, with loss of 41 per cent in body weight, corresponding to 50 per cent of the entire water content. De Almeida (1926) reported that in frogs the loss in body weight before death from dehydration varies with the rapidity of evaporation. With rapid desiccation, death may occur with a loss of only 10 per cent, while with slower experiments the loss may be 30 to 40 per cent. Further data on dehydration in the frog are given by Iizuka (1926) and Hug (1927). Data for comparison as to storage and loss of water in other species and in other forms of inanition are cited by Jackson (1925, 1929). These include the effects of exsiccation on various invertebrates and vertebrates.

The curves of body weight during dehydration in our test groups C and D (Fig. 1) show a more rapid loss at the beginning, with a progressively slower rate of decrease later. The rate of rehydration followed by test group D appears slower at first, but is more nearly uniform from the third to the 26th hour (58th hour of the experiment), when the weight ceased to increase. This group, therefore, did not return to their original body weights, but maintained a nearly constant body weight averaging 36.74 grams, or 8.06 grams below their original weight. This difference is apparently due chiefly to the partially unrestored loss of water from the lymph spaces. It is probably caused by the change in room temperature, which was 8° to 11° up to the beginning of the experiment, but 25° C. during the test. Ott (1924) found that frogs on transfer to cooler environment may absorb water (chiefly in the lymph spaces), which is lost within a few days when they are removed to warmer quarters.

Individual Organs and Parts

Fresh Weights.—The losses in fresh weights of the organs in the desiccated group C agree in general with the findings of Durig (1901). He reported that the musculature lost most heavily, but he did not measure the loss from the lymph spaces, which we find to be even greater. According to Durig, the brain and heart lost relatively less than any other organ; but our data indicate that the eyeballs, lungs and skeleton (organs not studied by Durig) lost relatively less fresh weight than the brain or heart. Durig also found that the kidneys, in spite of a marked decrease in water content, showed a relative increase in weight, which he ascribed to retention of insoluble urinary constituents. The kidneys in our desiccated group C lost only 16.7 per cent of their fresh weight, so that they gained in relative weight, as did all the other organs losing relatively less than the body as a whole.

Also in other species under conditions of dehydration, certain organs such as the brain, eyeballs, skeleton and heart maintain their organ weights remarkably well (in some species even increasing their weight under these conditions); while other organs such as the skin, muscles, stomach-intestines, liver and spleen lose a large proportion of their fresh weight. (See review of the literature by Jackson, 1925, 1929.) A similar tendency was found by Ott (1924) in the frog during inanition.

Ott found that the excess water absorbed when frogs were transferred to a cooler environment did not appreciably affect the weights of the various organs, excepting the lungs and fat bodies. In the present experiments, all the organs excepting the skin, spleen, liver and fat bodies (as shown in Table I) in the rehydrated group D had returned to nearly normal weight, and all excepting the heart and skeleton (Table III) had returned to within two per cent of the original normal water content. Data for the water content of the fat bodies are lacking however.

Loss of Solids.—A comparison of the figures for fresh organ weights in Tables I and II shows certain discrepancies between the desiccated test group and the rehydrated test group D. All the organs of test group D returned to within 8 per cent of the normal weight, excepting the skin, liver and spleen. As previously mentioned, these discrepancies are attributed chiefly to loss of solids during the experiment. The liver during the brief period of inanition probably lost much of its glycogen content, which could not be restored by the mere addition of water. This would account for the nearly normal relative water content of the liver in spite of its marked subnormality of weight. The skin in test group D started shedding (ecdysis) three to four days after beginning rehydration, which probably accounts for its marked subnormality in weight, although the relative water content had returned to nearly normal. Similarly the spleen may have lost solids (through escape or destruction of cells), which would account for the subnormality in weight in spite of nearly normal water content. The other organs apparently lost but little or no solids, as was noted above.

Water Content.—The average percentage of water content in the organs of our control group A (Table III) is in general agreement with Ott's figures for his normal control frogs. The differences are within 3 per cent, excepting the spleen, which is higher in his series, and the liver, skeleton and stomach-intestines, which are somewhat lower.

In his fasting frogs with loss of 40 per cent in body weight, the percentage of water content is usually increased, in contrast with our desiccated group C showing marked loss in water content.

Table IV indicates that the greatest absolute sources of water during desiccation in the frog are from the lymph spaces, muscles, skin, blood, liver, "remainder," and stomach-intestines, decreasing in the order named. In relative loss, however, the corresponding order is the lymph spaces, blood, skin, stomach-intestines and muscles.

The escaped blood of the test group C contained 77.33 per cent of water, or 11.64 per cent below the water content of the escaped blood in control group A (Table III). Yet the escaped blood of the rehydrated test group D had regained practically the same relative amount of water as the control group A. The markedly thickened blood of the dehydrated frog is therefore rapidly restored to normal. Durig found that the number of red corpuscles per cu. mm. was more than doubled in the frog by desiccation.

After recovery from desiccation, nearly all the organs of the rehydrated group D return to within two per cent of their normal water content (Table III), although insufficient water has been absorbed to restore the original body weight. Ott (1924) similarly noted that the excess water absorbed by frogs at lower temperature did not affect the weight or water content of the organs, excepting the lungs and fat bodies. The only exceptions in our rehydrated series at higher temperature are the skeleton and heart, which gain an excess water content of 2.39 and 3.10 per cent, respectively. The lymph spaces of the test group D, however, are estimated (as previously mentioned) to have remained 44 per cent subnormal in water content. It is evident that the changes in water content of the frog's body with fluctuations of temperature affect the amounts in the interstitial spaces rather than the water content of the tissues and organs.

It is not the purpose of the present paper to compare the findings of other investigators in various species, but it may be noted that, in general, the main storage depots of water in the frog, aside from the subcutaneous lymph spaces, appear to correspond in general to those found in mammals. The amphibian is more tolerant of dehydration, however, and can survive relatively greater losses. Moreover, the water in the amphibian organism is stored in a more labile form, so that it can be more readily removed and restored. Data for comparison are cited by Jackson (1929).

SUMMARY

The principal results of the present investigation may be summarized briefly as follows:

1. Male frogs (*Rana pipiens*) weighing about 44 grams were able to recover after desiccation with average loss of about 43 per cent of their body weight or about 51 per cent of their total water content.

2. The eyeballs and lungs of the desiccated frogs remained nearly unchanged in average fresh organ weight, and the skeleton and brain lost less than 8 per cent. The kidneys, heart, spinal cord, testes, stomach-intestines and muscles formed a group losing moderately (16–30 per cent), but relatively less than the body as a whole. The skin, tongue, spleen, liver and (escaped) blood lost 43–81 per cent, or relatively more than the body as a whole.

3. The average percentage of normal water content in the entire body was 82.14 per cent (range 80.21–84.28) at temperature of 8 to 11° C.

4. In the normal organs the average percentage of water content ranged from 58.13 in the skeleton to 88.97 in the escaped blood.

5. The decreases in percentage of water content in the desiccated frogs averaged approximately as follows: skeleton 0.2, heart 1.8, eyeballs 2.5, brain 2.6, liver 4.0, spinal cord 4.0, spleen 4.3, lungs 4.6, "remainder" 5.9, kidneys 6.3, testes 7.5, muscles 7.6, tongue 9.3, stomach-intestines 9.8, skin 10.2, and escaped blood 11.6.

6. The average absolute amount (grams) of water given up by the various organs or parts of the desiccated frogs was approximately as follows: lymph spaces 8.5, muscles 5.0, skin 2.3, blood 1.1, liver 0.8, "remainder" 0.3, stomach-intestines 0.2, tongue 0.2, skeleton 0.1. The remaining organs each lost less than 30 mgm. of water.

7. In the rehydrated frogs, all the fresh organs with the exception of the skin, spleen and liver regained nearly normal weight (within 8 per cent). In all the rehydrated organs, except the skeleton and heart, the water content returned to within 2 per cent of the norm. However, the amount of water in the interstitial lymph spaces remained 44 per cent subnormal, probably due to the increased temperature of the room.

8. The following peculiarities were noted in the rehydrated organs: The fresh weight of the skin remained 18 per cent subnormal, apparently due to ecdysis. The fresh weight of the spleen was 22 per cent subnormal, probably through escape or destruction of cells. The liver weight remained 39 per cent subnormal, probably through loss of glycogen. The relative water content in each of these three organs in the rehydrated group was found to be within 0.9 per cent of the normal.

9. In general, the main storage depots of the frog, aside from the lymph spaces, appear to correspond in general to those found in mammals. The amphibian is more tolerant of dehydration, however, and

can survive relatively greater losses. Moreover, the water in the amphibian organism is stored in more labile form, so that it can be more readily removed and restored.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

II. THE INFLUENCE OF pH, TEMPERATURE, AND OXYGEN TENSION ON HEMOLYSIS BY HYPOTONIC SOLUTIONS

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I

In the first paper of the present series (Jacobs, 1930), some of the more important advantages of the erythrocyte as material for the study of cell permeability were mentioned. At the same time it was pointed out that because of its peculiar sensitiveness to certain environmental factors whose effects upon ordinary cells are much less noticeable, the erythrocyte has acquired an undeserved reputation for variability in its osmotic behavior. In the present paper, the nature and magnitude of the effects of three such factors, namely, pH, temperature and oxygen tension, will be considered. It will be shown that at least two of them have for the erythrocyte an importance entirely out of proportion to that observed in the case of other cells and that their neglect in osmotic studies on this type of material is certain to lead to serious difficulties.

A survey of the literature shows that the importance of the first of the three factors was recognized by Hamburger, the pioneer worker in the field of osmotic hemolysis, whose numerous earlier studies are conveniently summarized in his book, "Osmotischer Druck und Ionenlehre" (1902). Hamburger, to be sure, did not distinguish clearly between the titratable acidity or alkalinity of a solution and its true reaction, now commonly expressed as pH; but he did, nevertheless, show conclusively that changes of the blood in the acid direction, whether produced by carbon dioxide or by other acids, result in a lowered osmotic resistance of the erythrocytes and that changes in the alkaline direction have the opposite effect. He correctly associated these changes in resistance with the volume changes, observed by von Limbeck (1895), which occur under the same conditions. The volume changes he interpreted in turn as being primarily of an osmotic nature, resulting from changes in the amounts of base bound by the cell proteins and from a certain type of ionic interchange between the cell and its surroundings (1902, pages 307 and 335). In its details, Hamburger's theory was rather indefinite and in several respects it has proved to be entirely erroneous, but it does nevertheless foreshadow to some extent the most modern views on the subject.

Among workers who followed Hamburger, there was for a time a tendency to abandon the osmotic explanation of the effects of pH changes and to attribute these effects to more or less vague colloidal phenomena. Haffner (1920), for example, in a study in which actual pH values are mentioned (for most of the values in question see Jodlbauer and Haffner, 1920), and which in this respect represents an advance over the work of Hamburger, concludes that, "Da es sehr unwahrscheinlich erscheint, dass der osmotische Druck der im Zellinnern in wahrer Lösung befindlichen Substanzen durch Verschiebung der H-Konzentration erhebliche, die obigen Befunde erklärende Änderungen erfährt, so folgt, dass für das Quellungsverhalten der Zelle als Ganzes der Quellungszustand—also der Ladungszustand—gewisser Zellkolloide von ausschlaggebender Bedeutung ist." Similar views have been expressed by other workers.

More recently the osmotic theory of the effect of pH on the volume of erythrocytes (from which its application to problems of hemolysis is an easy step) has again been brought forward, this time in a far more definite and satisfactory form than previously, in the very important papers of E. J. Warburg (1922) and of Van Slyke, Wu, and McLean (1923). For a somewhat briefer discussion of the theory in question Van Slyke (1924, 1926) may be consulted. Not only did this theory provide a plausible theoretical explanation of the known facts but the observed and predicted magnitudes of the effects in question, of which that on cell volume is the one which concerns us here, were found to be in good quantitative agreement. So generally satisfactory has this theory proved to be that it is most surprising that so little account of it has as yet been taken by persons interested primarily in problems of hemolysis. One of the purposes of the present paper is to point out its general applicability to such problems.

The second of the three factors under consideration, temperature, was also studied to some extent by Hamburger (1902, page 172), but with results which, probably because of the crudeness of his methods, were unsatisfactory. He found almost no measurable differences in the degrees of hemolysis produced by hypotonic solutions at 0°, 14° and 34° C. Such slight differences as he obtained seemed to indicate, if anything, slightly more hemolysis at higher than at lower temperatures, a result in the opposite direction from that observed when more accurate methods are employed.

A much more satisfactory piece of work is that of Jarisch (1921), in which a very clear and regular decrease in degree of hemolysis with rising temperature was obtained for a number of species of mammals. Neither the theory offered by Jarisch to account for this effect, however, nor the evidence upon which he based it are very convincing, and it seems much more plausible to look for an explanation along the same lines as those already shown to be useful in the case of pH effects. While the indirect osmotic influence of temperature upon the volume of the erythrocyte has apparently received only incidental attention, it is in all but its details included in the general theory of such volume changes.

Concerning the effect of the third factor, oxygen, on hemolysis, little information is available. Ordinary "fragility" studies are of very questionable value for reasons which will be discussed later. Strangely enough, Hamburger, whose own work had done so much to establish the importance of carbon dioxide as a factor capable of influencing hypotonic hemolysis, at times appeared to attribute the increased resistance of the erythrocytes from defibrinated blood solely to an increase of oxygen (for example, 1902, page 172), seemingly forgetting the simultaneous loss of carbon dioxide. It is obvious that conclusions of value can be drawn from such experiments only if the effects of the different variables are properly separated, and this appears not to have been the case in the work so far published in this field. On the other hand, the effects of oxygenation of the hemoglobin upon cell volume have been adequately dealt with by Van Slyke, Wu and McLean (1923) and by Henderson, Bock, Field, and Stoddard (1924), and the application of the results of these workers to problems of hemolysis is obvious.

Summarizing our present knowledge of the subject, it may be said that from the experimental point of view the general effects of pH and temperature upon the degree of osmotic hemolysis are known, at least qualitatively, even though they are only too frequently neglected in practice. The effect of oxygen tension is known with less certainty. On the other hand, the effects of all of these factors upon cell volume

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can be predicted theoretically from the known properties of the erythrocyte, of which the base-binding power of its hemoglobin is one of the most important; and the calculated and observed volumes have been shown in the case of pH to be in good agreement (Warburg, 1922; Van Slyke, 1924). It remains to determine how far the simple and definite theory of osmotic volume changes is capable of accounting for the effects upon hemolysis of the three factors in question and how far less precise theories of "colloidal behavior" must be considered. Only to the extent that the first type of explanation can be shown experimentally to be inadequate will it be necessary to invoke the second.

II

Before the presentation of the experimental results, it may be pointed out that the method of hemolysis, when properly employed, is perhaps the most delicate method at present known for investigating the osmotic volume changes of cells. This highly desirable characteristic of the method is, rather paradoxically, due to a property of the biological material with which it is employed which is commonly thought of only as a disadvantage, namely,—its variability. As is well known, the erythrocytes in a given sample of blood form a highly heterogeneous population with respect to their resistance to osmotic swelling. In Fig. 1 is represented the distribution of cells of different resistances in a

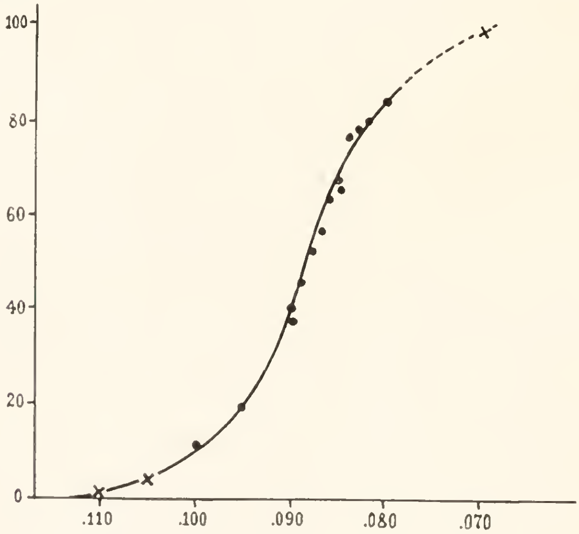

FIG. 1

FIG. 1. Variation in osmotic resistance of erythrocytes of the ox. Ordinates represent percentages of hemolysis; abscissæ, times in minutes. Conditions of the experiment as mentioned in the text.

sample of ox blood. The data were obtained under the standard conditions of 20° C., pH 7.4, the oxygen tension of room air, an exposure to the solutions for an hour with stirring and the use of such small quantities of blood that the composition of the solution does not undergo any appreciable change during the course of hemolysis. The points represented by circles, which were obtained by the method previously described (Jacobs, 1930), and those represented by the two lower crosses, which were obtained by the other author by a new method to be described elsewhere, fall very satisfactorily upon the same smooth curve. The uppermost cross is based upon a direct microscopic examination of the suspension.

The cause of the osmotic variability of the erythrocytes is not definitely known. It may be associated with differences in the properties of the membranes of different cells, either in resisting stretching or in becoming permeable to hemoglobin when stretched. Another possibility was suggested by Hamburger, namely, that different cells contain different amounts of substances such as the materials of the stroma and hemoglobin which occupy a part of the cell volume without themselves undergoing osmotic volume changes. The higher the percentage of such substances the less the swelling of the cell as a whole in a given hypotonic solution would be and the less, therefore, its tendency to undergo hemolysis.

Whatever factor or factors may be responsible for the osmotic variability of the erythrocytes, the important fact is that these cells are so numerous (approximately 250,000,000 in an ordinary drop of blood) that, on the one hand, successive samples from the same lot of blood are almost identical and, on the other, the variation is practically continuous, *i.e.,* there are represented an almost infinite number of degrees of osmotic resistance. Under these conditions, the variability of the cells instead of being a source of error becomes an advantage by rendering it possible to compare with a high degree of accuracy the osmotic properties of solutions whose concentrations differ only very slightly.

A fair idea of the degree of accuracy obtainable with the method of osmotic hemolysis without the employment of any unusual precautions is illustrated in Fig. 2. In this figure smooth curves have been drawn through the points transferred from the original kymograph record (see Jacobs, 1930). The slightly buffered NaCl solutions employed differed in concentration by 0.001 M. With one or two slight discrepancies the curves form a very regular series. It would obviously be possible in the most sensitive part of the range of the instrument without further precautions than those here taken to distinguish the osmotic effects of solutions differing from one another by 0.0002 M or less, *e.g.,* the effects of 0.0880 M and 0.0882 M NaCl. Such a difference in concentration would produce in an osmotically perfect system a volume change of less than 0.25 per cent and in an erythrocyte in which the volume of the non-liquid part of the cell has been estimated at from 35 or 40 per cent (Ege, 1921) to 65 or 70 per cent (Gough, 1924; Krevisky, 1930) a visible change for the cell as a whole of only between 0.1 and 0.2 per cent. Differences of this order of magnitude, which are by no means the smallest that could with care be measured, could probably not be dealt with successfully by any other method. Even the large spherical *Arbacia* egg, the most favorable cell in certain respects for osmotic studies which has yet been employed, does not permit the accurate measurement of volume changes ten times as great.

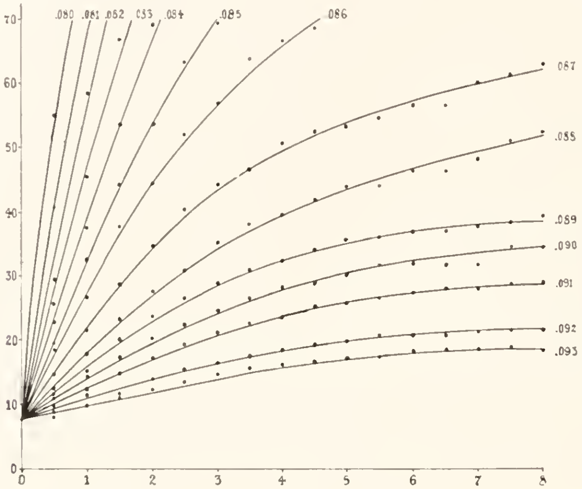

FIG. 2

FIG. 2. Effect of concentration on the rate of hemolysis of ox erythrocytes at 20° C. and pH 7.4. Ordinates represent scale readings of the instrument and abscissæ times in minutes.

It follows that the hemolysis method under favorable conditions is one of great delicacy. As with other delicate methods, however, the effects of disturbing factors are correspondingly serious. Some of these factors will now be considered.

III

The most important factor theoretically and the most difficult one to control practically is the pH of the medium. It is safe to say that more errors have been caused in studies of osmotic hemolysis by neglect of this factor than of any other. Where a relatively large quantity of blood is used with an unbuffered salt solution, the resulting pH will be determined chiefly by that of the blood, which in turn will vary greatly according to the amount of CO_2 which has been allowed to escape, etc. If very small quantities of blood are employed the case is no better, since now the final pH will be more strongly influenced by that of the solutions used, which because of absorption of carbon dioxide from the laboratory air, etc. may be expected, in the absence of buffers, to vary considerably. Under such conditions, even a breath from the experimenter at the wrong time may completely ruin an experiment. It is evident, therefore, that to obtain accurate results it is important on the one hand to use very small quantities of blood and on the other to work with buffered solutions of known pH. These precautions have been used throughout this work.

In carrying out experiments upon the effects of pH changes in which concentration differences of 0.0002 M are significant, the usual phosphate buffers are not entirely satisfactory. Solutions of Na_2HPO_4 have considerably higher osmotic pressures than those of NaH_2PO_4 of equal concentration, and mixtures of such solutions in different proportions therefore involve an important variable in addition to pH. Theoretically, it is possible to find a concentration of HCl which when mixed with a solution of Na_2HPO_4 will cause no appreciable change in osmotic pressure, the resulting volume changes just balancing those in dissociation; practically, however, it is difficult to be certain that pH standards made up in this way are exactly equivalent, since the ordinary freezing point method for osmotic pressure determinations is not sufficiently delicate for the necessary tests. It has proved better in practice, therefore, to use a solution containing a fixed amount of $NaHCO_3$ and to vary the pH by adding different amounts of CO_2, a substance which not only causes no appreciable volume changes in the solution itself but which distributes itself so rapidly between the solution and the cells that its direct osmotic effect upon the latter can be considered to be zero.

The disadvantages of the bicarbonate-CO_2 buffer system are, first, that because of the volatility of the CO_2 the experiments must be carried out in tightly stoppered bottles in which stirring is somewhat difficult to accomplish and from which samples cannot be removed at will; and, second, that the highest CO_2 tensions involve a simultaneous decrease in oxygen tension which in itself has indirect osmotic effects. As will be shown later, however, these effects are small in magnitude and, on the whole, the advantages of the $NaHCO_3$–CO_2 buffer system greatly outweigh its disadvantages for experiments such as the one about to be described.

The procedure employed in this experiment was as follows. A mixture of $M/1$ NaCl and $M/1$ $NaHCO_3$ (4:1) was diluted to 0.12M, 0.11 M, etc., these concentrations referring to the two salts taken together. It is permissible to combine the concentrations of the salts in question because of their great osmotic similarity. Taking first any chosen concentration, a small part of the solution was almost saturated with CO_2. This saturated solution was then mixed with the unsaturated solution in different proportions. For most of the mixtures the amount of CO_2-containing solution was so small relatively that the oxygen tension of the resulting combination was not greatly changed; in the solutions of lowest pH, however, it was considerably reduced. This reduction might have been largely prevented by the use of pure oxygen in conjunction with the carbon dioxide, but in view of the comparatively slight osmotic effects of even fairly large variations in oxygen tension (see Section V), this refinement was not considered necessary.

As each solution was prepared, two carefully measured drops of defibrinated blood were introduced into 50 cc. of it in a closed vessel. As soon as thorough mixing had been accomplished a 30 cc. glass-stoppered bottle was quickly filled with the suspension of erythrocytes through a glass tube without unnecessary exposure to the air. The bottle, completely filled and stoppered, was placed in a water-bath at 20° C. and rocked gently for one hour. A small glass rod in the bottle, kept in continuous movement by the rocking of the latter, prevented the erythrocytes from settling to the bottom. Immediately after the bottle had been filled a pH determination was made upon a part of the remaining solution with a quinhydrone electrode designed to prevent escape of CO_2. After a series of different pH values had been studied for one concentration a second concentration was similarly employed and so on until the entire range had been covered.

The results of this experiment are represented in Fig. 3 in which percentage of hemolysis is plotted against pH, and concentrations are represented by contour lines. It will be noted that starting at any given point the percentage of hemolysis may be increased either by diminishing the concentration or the pH, and decreased by changes in the reverse direction. Furthermore, it is possible, by changing the two variables in opposite directions, to secure an exact balancing of their effects. For example, by following the level of 50 per cent hemolysis from the contour line for concentration 0.110 M to that for 0.100 M it is seen that a concentration difference of 0.010 M is here equivalent to a pH difference of 0.45. Considering the figure as a whole, there is seen to be some variation in the pH-concentration relation, though the order of magnitude remains the same, being in the vicinity of 0.5 pH units for a concentration difference of 0.010 M. A comparison will later be made between this observed order of magnitude and that predicted by the osmotic theory of pH effects.

One further point is of interest, namely, that if, for example, a concentration change from 0.110 to 0.100 is equivalent to a pH change of approximately 0.5 and if, as is the case, a concentration difference in this region of 0.0002 M or a concentration ratio of 0.9998 has a visible effect upon the observed degree of hemolysis, then a pH change of 0.01 should have approximately the same effect. In other words, uncontrolled pH differences of more than this magnitude may be expected to be a source of error in experimental work.

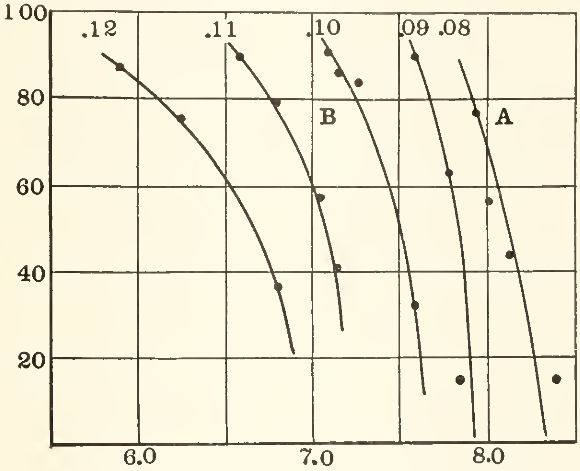

FIG. 3

FIG. 3. Effect of pH on osmotic hemolysis. Ordinates represent percentages of hemolysis; abscissæ, pH values; contour lines, concentrations in mols per liter.

IV

Experiments on the effects of temperature on the observed degree of hemolysis are considerably easier to carry out than those of pH because a single phosphate buffer system may be used throughout the entire series. It is true that the necessary differences in the degree of dilution of the buffer salts in such experiments will produce slight pH changes, but the effects of these changes are almost negligible in comparison with the large concentration differences involved.

In the experiment about to be described, which is a typical one chosen from several of the same general type, the erythrocytes were those of the cat. The stock salt solution employed consisted of $M/_1$ NaCl and $M/_1$ Na_2HPO_4 in the proportion of 14 to 1, with the pH reduced to approximately 7.0 by means of concentrated hydrochloric acid so as to give upon dilution a pH in the vicinity of 7.4. A mixture of this sort was, of course, not exactly equivalent osmotically to $M/_1$ NaCl, but the difference was not great and, in any case, it was relative rather than absolute concentrations which were of importance in this experiment. From the stock solution the necessary dilutions were made, treating the original solution as $M/_1$. The pH values for the various dilutions, as determined with the quinhydrone electrode both before and after the addition of the blood, had in previous experiments been found in all cases to vary only very slightly from a value in the vicinity of 7.4. In this connection, it may be mentioned that in making progressive dilutions of the stock solution the pH changes fairly rapidly at first from its initial value of approximately 7.0, but by the time concentrations such as those here employed have been reached, at which the pH is in the neighborhood of 7.4, the effect of further dilution is slight.

The blood in the proportion of two carefully measured drops to 50 cc. was introduced into the solutions, previously prepared and brought to the desired temperatures, which in the case of those at 0° C. and 20° C. were kept constant within 0.1°, and in the case of the others within less than 0.5° in water-baths. The solutions were gently and continuously stirred throughout the experiment. Determinations of the percentages of hemolysis attained were made at the end of one hour. This time is not sufficient for the establishment of complete equilibrium

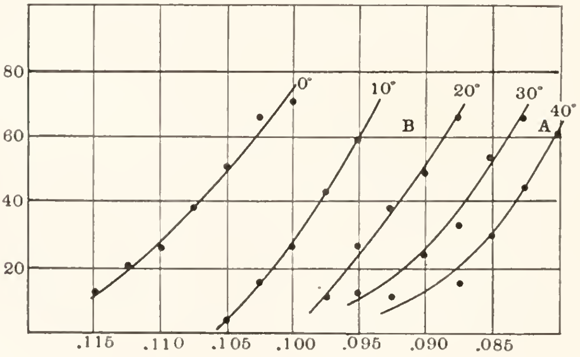

Fig. 4

Fig. 4. Effect of temperature on osmotic hemolysis. Ordinates represent percentages of hemolysis; abscissæ, concentrations in mols per liter and contour lines temperatures in ° C.

at 0° and 10° C., but the further changes at these temperatures are slight and the disadvantages of experiments of longer duration probably outweigh their advantages.

The results of the experiment are presented in Fig. 4. The percentage of hemolysis is here plotted against concentration, and contour lines are used to represent the different temperatures. It will be observed that for the region of 50 to 75 per cent hemolysis, where the erythrocytes are presumably fairly typical and where errors in the measurements are small, a change in temperature of 10° C. is exactly balanced by one in concentration from 0.0035 M to 0.0075 M. In other words, for the region in question, a concentration difference of 0.001 M is roughly equivalent to a temperature difference of the order of magnitude of two degrees. It follows, therefore, that if a visible influence upon the observed degree of hemolysis is exerted in this region by a concentration change of 0.0002 M, the same result should be obtained by a temperature change of less than 0.5°. In the light of this relation, it is not surprising that a lack of agreement is frequently found in experiments carried out at "room temperature."

Reference may here be made to the results obtained by Jarisch (1921). From his figures on page 256 it appears that in the case of the ox, for example, a concentration of 0.473 per cent NaCl at 40° C. was equivalent to one of 0.519 per cent at 15° C. The results for several species of mammals are presented graphically on page 257 of the same paper. It should be noted, however, that the method employed by Jarisch was unsatisfactory in two respects. In the first place, only fifteen to twenty minutes were allowed for the attainment of equilibrium and, in the second place, no account was taken of anything short of complete hemolysis (as judged by the eye). The use of more refined methods would almost certainly have given a continuous fall in the curves represented on page 257 to 0° C. instead of only to 10° or 15° C. Making allowance, however, for these and possibly other differences in technique and remembering that the concentrations in the experiments of Jarisch are expressed in percentages of NaCl rather than in mols per liter, it appears that his results were of the general order of magnitude of those here reported.

V

The effects of oxygenation upon the degree of hemolysis may next be briefly described. On the whole, they prove to be much smaller in magnitude than those produced by pH and temperature changes—in fact, their practical importance as a source of error must be very small since most laboratory solutions are in equilibrium with room air, and

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in such solutions hemoglobin will be almost completely oxygenated. Only if a deliberate effort were made to reduce the hemoglobin would effects of any important magnitude be expected.

Several experiments were tried at various times to determine the effects of oxygenation at constant pH upon the degree of hemolysis. The experiment here reported was a typical one. In it, ox blood was placed at 20° C. in a series of different dilutions of the buffered stock solution mentioned in Section IV. The degree of hemolysis observed at the various dilutions is represented by the solid circles in Fig. 5. After this preliminary standardization, two lots of 50 cc. each of the 0.085 M, 0.0875 M and 0.090 M dilutions were placed in separate vessels through which oxygen and hydrogen, respectively, were bubbled for ten minutes. At the end of this time two accurately measured drops of blood which had previously been introduced into the vessels but not mixed with the liquid were submerged in the oxygen-rich and oxygen-poor solutions. The gases were allowed to bubble slowly through the resulting suspensions for an hour, and then determinations of the degree

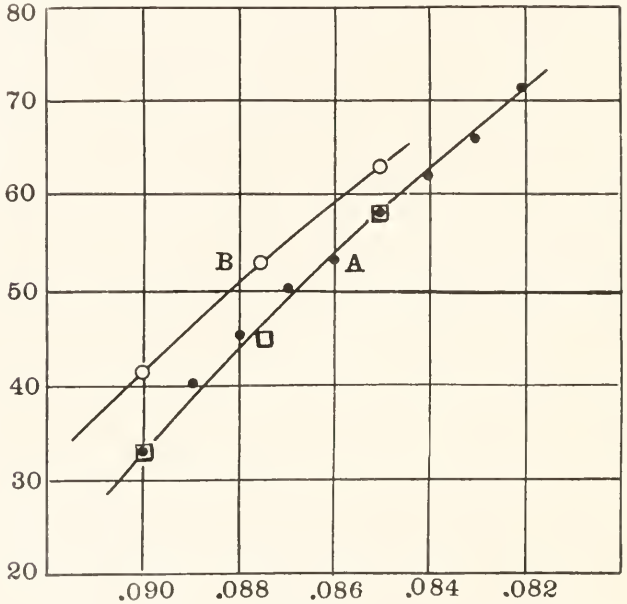

Fig. 5

Fig. 5. Effect of oxygenation on osmotic hemolysis. Ordinates represent percentages of hemolysis and abscissæ concentrations in mols per liter. The degree of oxygenation is represented as follows: open circles, complete reduction; solid circles, equilibrium with room air; squares, complete oxygenation.

of hemolysis were made in the usual way. The pH of the various solutions did not vary beyond the limits of error of the quinhydrone electrode determinations. The hydrogen used contained approximately 0.05 per cent of oxygen, so reduction of the hemoglobin was probably almost but not quite complete.

The degrees of hemolysis obtained under the different conditions are represented in Fig. 5 by the open circles for the reduced and by squares for the oxygenated blood. From the figure it is apparent that the maximum difference obtainable under these conditions is equivalent to one in concentration of approximately 0.0016 M. Otherwise expressed, by reduction of the hemoglobin the degree of hemolysis at the concentrations in question is increased by about five to eight per cent. Neither in this experiment nor in other similar ones were significant differences observed between the effects of room air and those of pure oxygen, which is what would be expected from the known character of the oxygen dissociation curve of hemoglobin.

VI

In the three sections immediately preceding this one there have been described and measured in terms of equivalent concentration changes the effects upon osmotic hemolysis of variations in pH, temperature, and oxygen tension. It remains to determine whether the magnitude of these effects is such as can be accounted for wholly or chiefly by osmotic differences within the cell resulting from changes in the base-binding powers of the hemoglobin. Van Slyke, Wu and McLean (1923) have treated at length from this point of view the effects of pH and, to some extent, those of oxygen tension upon the related problem of cell volume, but their equations apply to the more complicated situation where the cells are suspended in a relatively small volume of a protein-containing solution (serum) whose composition varies with that of the cells. We are concerned here with the simpler case, to which these equations are not directly applicable, of cells suspended in a protein-free salt solution whose volume is so great that its composition may for practical purposes be considered to be constant. It will be necessary, therefore, following in part the method of Van Slyke, to derive an equation applicable to this type of system by which the theoretical volume changes associated with changes in the base-binding powers of the hemoglobin may be estimated.

The substances of which account must be taken in the derivation of the equation are the cations of the system (chiefly K° and Na°) together designated as *B* (base), and the anions exclusive of *Hb′* (chiefly

Cl' and HCO_3') together designated as A', hemoglobin ions Hb' and hemoglobin uncombined with base, together designated as Hb and base bound by hemoglobin, designated as BHb. The question of the degree of oxygenation of the hemoglobin need not be raised until later. Following Van Slyke, the symbols $(B)_c$, $(A)_c$, $(B)_s$, $(A)_s$, etc. will be used to refer to the amounts of B and A in the cells and solution, respectively, in a given quantity of the suspension and $[B]_c$, $[A]_c$, $[B]_s$, $[A]_s$, etc. to the corresponding *concentrations* expressed per unit amounts of water rather than per unit volume of solution, *i.e.*, $[B]_c = (B)_c/(H_2O)_c$. Van Slyke uses as his units the amounts of the substances other than water in milli-equivalents and of water in kilograms for one kilogram of blood. The resulting concentrations are therefore expressed in milli-equivalents per kilogram of water. In the present treatment, where relative results only are desired, the particular units employed are of no consequence as long as they are used consistently throughout.

With regard to the measurement of quantities and concentrations of hemoglobin, Van Slyke, in the absence of definite knowledge at the time his work was done of the molecular weight of this substance, used as an equivalent of hemoglobin the amount that combines with one mol of oxygen. This assumption leads to calculated osmotic pressures for hemoglobin which, in the light of our present knowledge, are probably too high. However, according to the equation given by Adair (1925, page 533), it would appear that the discrepancy is certainly much less than it would be if the true molecular weight of hemoglobin could be employed in the usual way for calculating the osmotic pressure of concentrated solutions. In view of this fact, and in the absence of any very definite knowledge concerning the exact contribution, which in any case is relatively small, of hemoglobin to the total osmotic pressure within the erythrocyte, we may without serious error continue to employ Van Slyke's convenient assumption, particularly in a case like the present one where comparative results only are desired.

In order that osmotic equality inside and outside of the cell may exist, which is known to be the case with the erythrocyte, whose delicate wall is incapable of supporting in either direction an excess of osmotic pressure, the following relation must hold between the concentrations of the various osmotically significant substances:

$$[B]_c + [A]_c + [Hb]_c = [B]_s + [A]_s \tag{1}$$

or otherwise expressed,

$$\frac{(B)_c + (A)_c + (Hb)_c}{(H_2O)_c} = \frac{(B)_s + (A)_s}{(H_2O)_s}. \tag{2}$$

Since the cell is impermeable to hemoglobin and to base but permeable to water, $(Hb)_c$ and $(B)_c$ will remain constant, while $[Hb]_c$ and $[B]_c$ will tend to vary. Indeed, the values of the latter quantities may be used as a measure of the volume of the "liquid" portion of the cell and indirectly of that of the cell as a whole, provided that information is available concerning the bulk of the "non-liquid" materials. The erythrocyte is known to be permeable to the osmotically important anions, so neither $(A)_c$ nor $[A]_c$ will be fixed but will in general vary in such a way that the Donnan ratio, $[A]_c/[A]_s$, will have the value determined by the other properties of the system. (The use of concentrations in place of activities introduces no great error; for the justification for assuming complete dissociation of the various salts involved —Van Slyke, Wu and McLean (1923)—may be consulted.)

If now in equation (2), $(B)_c - (BHb)_c$ be substituted for $(A)_c$, $2(A)_s$ be substituted for $(B)_s + (A)_s$ and the terms be suitably rearranged we obtain:

$$\frac{(\mathrm{H_2O})_s}{(\mathrm{H_2O})_c} = \frac{2(A)_s}{2(B)_c - (BHb)_c + (Hb)_c}. \tag{3}$$

Similarly, by substituting in equation (2) $(A)_c + (BHb)_c$ for $(B)_c$, $2(A)_s$ for $(B)_s + (A)_s$, and rearranging, we have:

$$\frac{2(A)_s}{(\mathrm{H_2O})_s} = \frac{2(A)_c}{(\mathrm{H_2O})_c} + \frac{(BHb)_c + (Hb)_c}{(\mathrm{H_2O})_c}. \tag{4}$$

Dividing both sides of equation (4) by $2(\mathrm{A})_s/(\mathrm{H_2O})_s$, substituting the value of $(\mathrm{H_2O})_s/(\mathrm{H_2O})_c$ from equation (3) and remembering the definitions of $[A]_c$ and $[A]_s$ and of the Donnan ratio, r, we have finally:

$$r = 1 - \frac{(BHb)_c + (Hb)_c}{2(B)_c - (BHb)_c + (Hb)_c}. \tag{5}$$

Up to this point Van Slyke, Wu and McLean have been followed in principle, and equation (5) is the same as their equation (14) except that $(BP)_s$, the base bound by the protein in the solution, is here zero, there being no protein present in the solution. The base bound by protein in the cell is also here designated as $(BHb)_c$ instead of as $(BP)_c$. Beyond this point a somewhat different treatment of the problem has been found convenient.

If under any given conditions of pH, temperature and oxygenation, the amount of base bound by unit amount of hemoglobin be represented by F, we may write $F(Hb)$ instead of (BHb) and equation (5) becomes:

$$r = 1 - \frac{(Hb)_c\ (1 + F)}{2(B)_c + (Hb)_c\ (1 - F)}. \tag{6}$$

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Substituting in equation (1) $r[A]_s$ for $[A]_c$ and $F[Hb]_c$ for $[BHb]_c$, we obtain:

$$[Hb]_c = \frac{2[A]_s\ (1 - r)}{1 + F}. \tag{7}$$

Introducing into (7) the value of r from (6):

$$[Hb]_c = \frac{2[A]_s\ (Hb)_c}{2(B)_c + (Hb)_c\ (1 - F)}. \tag{8}$$

As mentioned above, the concentration of hemoglobin $[Hb]$ is determined by the amount of water in some given quantity of cells, so that $1/[Hb] = kW$, where W is the water in one cell and k is a constant whose value need not be determined since it will subsequently be eliminated. Representing the ratio $(B)_c/(Hb)_c$ by R and substituting C, the concentration of the salt in the external solution, for $[A]_s$, we obtain from equation (8):

$$\frac{1}{[Hb]} = kW = \frac{2R + 1 - F}{2C}. \tag{9}$$

If C is kept constant, it can be seen that the theoretical effect on the amount of water in a single cell caused by a change of pH, temperature, or oxygen tension will be governed by the extremely simple relation:

$$\frac{W_1}{W_2} = \frac{2R + 1 - F_1}{2R + 1 - F_2}, \tag{10}$$

where W_1 and W_2 are the amounts of water in the cell and F_1 and F_2 are the amounts of base bound by a unit amount of hemoglobin under any two chosen conditions, R as defined above representing the ratio of base to hemoglobin within a single cell or any given quantity of cells. If, on the other hand, F is kept constant and C is varied, we have the usual osmotic equation,

$$\frac{W_1}{W_2} = \frac{C_2}{C_1}. \tag{11}$$

(F is actually not entirely independent of C, but for the small concentration differences here involved it may be considered to be.)

VII

It is now possible to determine whether or not the observed effects on osmotic hemolysis of the factors pH, temperature, and oxygen tension are of the order of magnitude of those predicted by the simple theory developed in the preceding section. A convenient method for making the comparison is to convert equation (9) into the following form:

$$\frac{W_1}{W_2} = \frac{2R + 1 - F_1}{2R + 1 - F_2} \cdot \frac{C_2}{C_1}. \qquad (12)$$

C and F have here both been allowed to vary simultaneously, it being assumed that these variables are independent of one another, which under the conditions in question is approximately true.

Two observed points on a line of equal hemolysis are now chosen. By the osmotic theory equal percentages of hemolysis indicate the entrance into the cells of equal quantities of water, *i.e.*, under these conditions $W_1 = W_2$, and the resulting value of the right-hand side of equation (12) should be unity. If, therefore, on substituting in equation (12) the appropriate numerical values of C and F for the two chosen points together with the proper value of R for the blood in question, the expression becomes equal to, or nearly equal to one, the observed results may be said to be in agreement with the theory. Any considerable departure from this value, on the other hand, will indicate inadequacy of the theory.

Concerning the values of C there is no difficulty. The necessary values of R and F, however, cannot at present be obtained with the same degree of certainty, since the work here reported was done on the blood of two species, the ox and the cat, for which exact data concerning these quantities are apparently not yet available. Indeed, even if such data had been published, it is not likely that different animals of the same species would fail to show some individual differences. In view of the fact, however, that various simplifying assumptions have been made in the derivation of the equations, which at best are only approximate, and the further fact that, as far as is known, the bloods of different mammals resemble one another fairly closely, it would seem to be permissible to take from the literature such data as are at present available, expecting no more than that the calculated and observed results may perhaps prove to be of the same order of magnitude.

With regard to R, in the absence of any more definite information concerning the bloods actually used, we may take as a plausible value that given for the horse by equation (15) of Van Slyke, Wu and McLean (1923), namely, 6.0. This value will be used in all of our calculations.

Before the magnitude of the theoretical pH effects can be estimated, it must be noted that the pH values used in the calculations of the base-binding power of hemoglobin are those of the interior of the cell, while those actually observed in the hemolysis experiments are those of the solution. The relation between the two by the Donnan principle is:

$$\text{pH cell} - \text{pH solution} = \log r,$$

where r can be obtained by means of equation (6), preferably first converted to the simpler form:

$$r = 1 - \frac{1 + F}{2R + 1 - F}.$$

To avoid the mathematical difficulties involved in attempting to determine pH cell from pH solution, it is convenient to work in the opposite direction, assuming a number of values of pH cell and calculating the corresponding values of pH solution. These latter values may then be plotted and a smooth curve drawn through them from which the desired values of pH cell may readily be obtained for any observed value of pH solution. In the calculations which follow this has been done.

Fairly extensive data for the calculation of pH effects are available for the blood of the horse at 38° C. Temporarily disregarding the difference in temperature, we may use for the calculation of F the equation given on page 152 of the paper by Hastings, Van Slyke, Neill, Heidelberger and Harington (1924) with the substitution of the necessary constants from Table XXIII for horse hemoglobin with a cation concentration of 145 mM.

Referring now to Fig. 3, we may choose any two points such as A and B which represent the same degree of hemolysis, for example, 75 per cent at pH 8.0 and pH 7.0 respectively. These particular points have been selected in order to keep within the range actually studied by Warburg (1922) and by Van Slyke, Wu and McLean (1923) and at the same time to take advantage of the region where our method of measuring hemolysis (Jacobs, 1930) is most accurate. The concentrations represented are 0.107 M and 0.079 M respectively. Remembering that the values of cell pH corresponding to solution values of 7.0 and 8.0 are 6.92 and 7.75, and assuming complete oxygenation of the hemoglobin at the tension of room air, we obtain by calculation $F\ 7.0 = 0.97$ and $F\ 8.0 = 3.34$. Substituting these various values, together with that of R in equation (12) we have finally

$$\frac{W'_A}{W'_B} = \frac{12 + 1 - 3.34}{12 + 1 - 0.97} \cdot \frac{0.107}{0.079} = 1.09.$$

The agreement between observation and theory is therefore seen to be fairly close. It may be noted that while the closeness of this agreement will vary somewhat with the positions of the selected points, A and B, owing perhaps to the influence of unknown factors of secondary importance, the points actually chosen are by no means the most favorable that could have been selected for the theory. Furthermore, over a considerable range of pH and concentration, the observed and predicted results are at least of the same order of magnitude. This, under the circumstances, is all that reasonably can be expected.

The calculation just made involves a temperature differing considerably from the one at which the observations were made. It is not likely, however, if direct observations on the value of F at 20° C. were available, that their use would greatly change the ratio in question. According to Stadie and Martin (1924), the effect of temperature on the base-binding power of hemoglobin is exerted not upon the buffer value of the hemoglobin but only upon its isoelectric point. In other words, in the equation for reduced hemoglobin

$$[BHb] = \beta_R[Hb] \quad (\mathrm{pH} - \mathrm{pI});$$

a change of temperature causes a change in pI but not in β_R. Conditions for oxygenated hemoglobin are similar, though there is a very slight departure within the range in question from a simple linear relationship. Under these circumstances, in correcting our calculations so as to make them apply to 20° C., both numerator and denominator of the fraction:

$$\frac{2R + 1 - F_1}{2R + 1 - F_2}$$

will be changed in such a way that the value of the fraction itself is little altered. In other words, it appears to be approximately though not exactly true that a given pH change produces the same relative volume change at different temperatures.

The question last considered anticipates to some extent the discussion of the effects upon hemolysis of temperature changes at constant pH. For data upon the base-binding powers of hemoglobin at different temperatures we have used Fig. 1 of the paper by Stadie and Martin to which reference has already been made. Since the temperatures are here 38° C. and 15° C. we have selected as our own points for comparison A and B in Fig. 4 representing these two temperatures and a degree of hemolysis of 60 per cent. This percentage is as near to the region of the greatest accuracy of our instrument as the rather small amounts of blood used in this experiment permitted us to go. The concentration values are 0.081 M and 0.092 M, respectively. Since the relation between the pH of the cell and that of the surrounding medium is almost the same at a solution pH of 7.4, which is that which we employed, and a blood pH of the same value, we have taken our figures directly from the graphs of Stadie and Martin without further calculations.

Considering first the figures for reduced blood, which are based upon actual observations, we find $F\ 38° = 2.6$ and $F\ 15° = 1.0$. Substituting in equation (12), we have

$$\frac{W_A}{W_B} = \frac{12 + 1 - 2.6}{12 + 1 - 1.0} \cdot \frac{0.092}{0.081} = 0.98.$$

If, instead of the observed figures for reduced blood, we take the calculated ones for oxygenated blood, we have

$$\frac{W_A}{W_B} = \frac{12 + 1 - 3.2}{12 + 1 - 1.6} \cdot \frac{0.092}{0.081} = 0.98.$$

Finally, we may use the theoretical effect upon pI calculated by Stadie and Martin from the heat of ionization of hemoglobin. They estimate that a change from 38° to 20° causes a change of pI for reduced blood of approximately 0.3 in the alkaline direction. Taking, therefore, values of pI of 6.8 and 7.1 for the two temperatures in question, and one of 2.8 for β_R we have for F, *i.e.*, for $[BHb]/[Hb]$, $F\ 38° = 1.68$ and $F\ 20° = 0.84$. The concentrations for 60 per cent hemolysis for these two temperatures taken from our own data are 0.081 M and 0.089 M, respectively. Substituting these values as before in equation (12) we have

$$\frac{W_A}{W_B} = \frac{12 + 1 - 1.68}{12 + 1 - 0.84} \cdot \frac{0.089}{0.081} = 1.02.$$

This calculation, strictly speaking, applies only to reduced hemoglobin, but the results would not be very different under conditions of partial or complete oxygenation.

Finally, the factor of oxygenation at constant temperature and pH may be considered. Our own results upon hemolysis indicate a comparatively small osmotic effect of this factor. For the calculated effect at 38° the equation of Hastings, Van Slyke, Neill, Heidelberger and Harington (1924) already referred to may be used. As mentioned above, the difference in temperature in the two cases, while unfortunate, may be expected to have only a minor effect on the calculated result. At the pH of our experiment, namely 7.36, corresponding to an intracellular pH of 7.23 we find $F_0 = 1.90$ and $F_R = 1.22$. For points A and B in Fig. 5, which are typical, the concentrations are 0.0862 M and 0.0875 M. Substituting these various values in equation (12) we obtain:

$$\frac{W_A}{W} = \frac{12 + 1 - 1.90}{12 + 1 - 1.22} \cdot \frac{0.0875}{0.0862} = 0.96.$$

Otherwise expressed, the value of the water ratio W_0/W_R calculated from the theoretical base-binding power of the hemoglobin is 0.94; that calculated from the observed equivalent concentration difference is 0.98. These values are of the same order of magnitude and both suggest the likelihood of much smaller errors in experimental work from neglect of the factor of oxygenation than of either of the others considered.

From the calculations which have been given concerning the theoretical osmotic effects of changes of pH, temperature and oxygenation, it is apparent that the predicted results are at least of the order of magnitude of those actually observed. The agreement is better in some parts of the range of the variables considered than in others and is nowhere perfect, as might be expected considering the various simplifying assumptions made in the derivation of our equations, and the necessity for using data obtained for other species of animals under dissimilar conditions. The existence of factors other than the purely osmotic ones is by no means excluded by our experiments; indeed it appears to be likely. We believe, however, that such factors are probably of secondary importance and that until the possibilities of the osmotic principles here discussed have been exhausted by calculations involving the use of more accurate data than are now available it will be unprofitable to indulge in speculations concerning little-understood " colloidal properties " of the cell and its constituents.

VIII

It has been shown that the influence upon hemolysis of the factors pH, temperature and oxygen can be at least largely accounted for by the osmotic effects which they produce through changes in the base-binding power of the hemoglobin. The question arises why the erythrocyte appears to stand alone in its sensitiveness to such factors. Hemoglobin is not a unique substance in its ability to bind base, nor in the effect upon its base-binding power of at least the factors pH and temperature. The proteins of all cells share with it these properties and yet within physiological limits other cells appear to be only slightly affected by the factors in question. Indeed, in the case of pH effects, Lucké and McCutcheon (1926) over a very wide range of reactions were unable to obtain unmistakable evidence of volume changes in the case of uninjured cells.

It is perhaps not possible in the present state of our knowledge of cells other than the erythrocyte to give a complete explanation of these differences, but attention may be called to a number of significant facts.

In the first place, even if a cell such as the *Arbacia* egg were freely permeable to anions and not to cations as is the erythrocyte—which is probably not the case—equation (10) would suggest a reason for a lesser liability of the former type of cell to volume changes. Other things being equal, the larger the value of R in this equation the less will be the effect upon the ratio W_1/W_2 of a given change in F. Now the value of R for the *Arbacia* egg is probably several times as large as that for the erythrocyte. We have little information about the concentration of its cell proteins as compared with that of hemoglobin in the erythrocyte, but it is almost certainly lower; the concentration of base, on the other hand, must be, roughly, three times as great, since the isotonic NaCl solution for the *Arbacia* egg is approximately M/2 as compared with M/6 for the erythrocyte. A value of R five times as great for the *Arbacia* egg as for the erythrocyte would therefore not be beyond the bounds of probability. Furthermore, the change in F with change of pH depends upon the buffer value of the protein in question as defined by Van Slyke (1922). Now the buffer value of hemoglobin is approximately three times as high as that of some of the commonest proteins such as albumins and globulins (Hastings, Van Slyke, Neill, Heidelberger and Harington, 1924). The absolute values of F, of course, are also of importance, but they cannot as yet be compared very accurately for the two types of cells. In any case, other things being equal, the high buffer value of hemoglobin tends to favor the production of large osmotic effects by pH changes.

In the second place, it seems almost certain that external changes in pH have far less effect upon the internal pH of a cell such as the *Arbacia* egg than of the erythrocyte. In the latter the simple Donnan principle: $\text{pH}_{\text{internal}} - \text{pH}_{\text{external}} = \log r$, governs the relation between internal and external reaction, however the latter may be produced. At ordinary reactions the difference is not usually greater than 0.2 pH units. On the other hand, in the eggs of *Asterias* and *Echinarachnius*, which are probably very similar to that of *Arbacia*, the internal pH is not only normally very different from that of the surrounding sea water, *e.g.*, 6.8 as compared with 8.1 (Chambers, 1928), but is relatively independent of the pH of the latter. Only freely penetrating acids, such as carbonic, butyric, etc., or freely penetrating alkalies, such as ammonia, are able, without injury to the cell, to cause visible changes in the appearance of intracellular indicators or to affect the cleavage process in a decided manner. According to Chambers (1928), even acids and alkalies of the naturally penetrating type in moderate quantities do not noticeably change the reaction of uninjured protoplasm but affect only certain cell inclusions. On the whole, therefore, the opportunities for producing intracellular pH changes which alone could cause the type of changes described by equation (10) are very slight as compared with those existing in the case of the erythrocyte, and it is not surprising that the latter cell responds to such changes in a more or less unique manner.

Less is known about the effects of temperature than of pH on cells other than the erythrocyte. Obviously, however, the situation is very much more complicated in cells with a high rate of metabolism and a great variety of physiological activities than in the relatively simple erythrocyte. It would be expected, therefore, that in ordinary cells the effects predictable from equation (10) would be modified and obscured by others of a different nature. As far as it is legitimate to apply this equation, however, the remarks already made about the magnitudes of R and of F would hold in this case also. As for oxygen, the reasons for the unique behavior of the erythrocyte are so obvious that they require no special discussion.

IX

It appears from the foregoing discussion that the erythrocyte is a more or less unique type of cell. Its nature is such that apparently insignificant changes in environmental factors such as pH and temperature produce within it osmotic effects of a magnitude sufficiently great to be absolutely fatal to the securing of reproducible results by a method as sensitive as that of hemolysis.

It is now possible to appreciate the difficulties that have attended all work upon osmotic and perhaps, to a lesser extent, other types of hemolysis where the factors in question have been neither measured nor controlled. If it be true, as appears to be the case, that pH changes of 0.01 pH unit and temperature changes of 0.5° C. can produce measurable osmotic effects, what is to be said of the numerous papers which have been published upon the "fragility" of the erythrocytes under almost all conceivable normal and pathological conditions, in which in the absence of any information whatever concerning these factors an uncertainty of as much as 2.0 pH units and 10° C. or more may exist, and in which, in addition, there is frequently no assurance that the experiments have been sufficiently long continued to secure approximate equilibrium? It is disheartening to be forced to believe that a large part of the work in this much cultivated field is of very doubtful value, but there seems to be no escape from such a conclusion. The erythrocyte being what it is, attempts to use it for comparative osmotic studies without controlling especially the factors pH and temperature, and to a lesser extent oxygen tension, are scientifically in the same category with attempts to study volumes of gases without accurate regulation of temperature and pressure.

In conclusion, a point mentioned in the first paper of this series may again be emphasized. The erythrocyte is not in itself a highly unreliable and capricious form of material, as it is frequently believed to be. Such capriciousness as it may appear to possess is merely a reflection of the carelessness of the experimenter or of his lack of understanding of its true physiological nature. When used uncritically merely as so much "material" for experiments in the field of General Physiology, the erythrocyte is likely to be a source of much vexation to the experimenter. When treated in a manner appropriate to a highly specialized cell with unique functions and physiological properties, concerning which more exact information is already available than is the case with perhaps any other single type of cell, it is capable of yielding results of an extremely satisfactory character.

SUMMARY

1. By the method of osmotic hemolysis an attempt has been made to evaluate the indirect osmotic effects upon mammalian erythrocytes of changes in the pH, temperature, and oxygen tension of the surrounding medium.

2. The observed effects of these three factors, within the range considered, are of the order of magnitude of those predicted by the equation:

$$\frac{W_1}{W_2} = \frac{2R + 1 - F_1}{2R + 1 - F_2} \cdot \frac{C_2}{C_1},$$

where W_1 and W_2 are the amounts of water contained in an erythrocyte under two given conditions, F_1 and F_2 are the amounts of base bound by one equivalent of hemoglobin under the same conditions, C_1 and C_2 are the concentrations of the solutions in question and R is the ratio of base to hemoglobin within the cell. It is probable that the effects of the factors studied are primarily osmotic in nature, though smaller effects of a different sort are by no means excluded.

3. Certain differences between the osmotic behavior of the erythrocyte and that of other cells are discussed.

4. It is shown that pH changes of as little as 0.01 pH unit and temperature changes of as little as 0.5° C. may have a measurable effect upon the observed degree of hemolysis. It follows, therefore, that "fragility" tests and other osmotic studies upon erythrocytes in which these factors are not properly controlled are of little value.

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THE PIGMENT OF *VELELLA SPIRANS* AND *FIONA MARINA*

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I

The siphonophore, *Velella spirans Esch.* occurs in enormous numbers during the months of March and April in the Mediterranean and adjacent seas. Especially after two or three days of the sirocco, the enervating tropical wind from the south and southeast, these beautiful animals often appear in thousands. There are records of their being cast up on the beaches of Sicily in such large numbers as to form a broad blue line hundreds of yards in length. The animal consists of a flat ovoid plate of chitin surmounted by a triangular " sail " set perpendicularly to the plate and obliquely to its longest axis. The plate is usually 6 to 10 cm. long and 3 to 6 cm. wide. The sail rises about two cm. above the surface of the plate. Like all other members of the order, *Velella* is free-swimming and is carried by wind and current.

Velella is striking because of its color,—a very deep blue. The pigment is present in varying amounts in all cells of the ectoderm, endoderm and mesoglea on both oral and aboral surfaces of the disc. It is especially dense in the tissues immediately surrounding the pneumatophore on the aboral side, but in the blastostyles, dactylozooids and sail the pigment is more diffuse and imparts a pale blue color to those organs.

Associated with *Velella* are relatively large numbers of two molluscs, —the nudibranch *Fiona marina* Forsk., and the prosobranch *Janthina communis* Lam. Each of these species may be found by itself, but the presence of *Velella* invariably means the presence, in greater or less degree, of these two molluscs also. Both *Fiona* and *Janthina* were found feeding on *Velella*, and the eggs of each were found on partly devoured animals or on their chitinous skeletons. *Fiona* creeps readily at the surface inverted, but in a *Velella* swarm it is most often found clinging to the oral or aboral surface of the siphonophore and occasionally to the sail. Here it feeds on the soft tissues, beginning at the periphery usually, and abandoning the skeleton when all else has been eaten. When removed from *Velella*, *Fiona* is deep blue and its color is indistinguishable from that of *Velella*. Only the dorsal papillæ and the head contain the blue pigment, the ventral surfaces being in general whitish or pale yellow. The attempt was made to determine the relationship between the body color of *Fiona* and its feeding habits.

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In the laboratory the nudibranchs were removed from *Velella* and when starved, or fed on fish scraps, they rapidly lost the blue color. It was not possible to retain them alive until all the pigment had disappeared, but the loss of color was very pronounced after three days. When such animals were again permitted to feed on *Velella,* the deep blue color reappeared in the dorsal papillæ two to four hours after feeding began. The pigment was distributed through all the cells of those structures. On removing the nudibranch and starving it again, the color tended to disappear once more. Individuals, on being repeatedly starved and fed on *Velella,* showed the characteristic variation between light blue and the metallic blue of the food. In animals fed on colorless fish scraps for four days or more the color became very light blue or yellowish. *Fionæ* that were allowed to feed only on *Velella* retained their dense blue pigmentation.

Fragments of blue *Velella* tissue were found in the digestive tracts of all *Fionæ* examined immediately after feeding on *Velella.* In all cases the walls of the tract were impregnated with the same color. In these nudibranchs ramifications of the stomach and liver extend into the dorsal papillæ, and indeed food fragments of the same nature as those found in other parts of the tract were to be recognized in the lumina of the dorsal papillæ. There is no doubt, therefore, that the blue color of these *Fionæ* is derived directly from *Velella.* The pigment passes through the digestive tract and into the dorsal papillæ unchanged and thus imparts the characteristic blue color to the latter organs. *Fiona* has also been found on floating seaweed and debris. At low tide it may also be discovered under stones, where it feeds on small jelly-fishes and crustaceans. Unfortunately, it was not possible to obtain animals from these habitats for comparison. No function can be ascribed to the pigment in *Fiona.*

There is present in *Janthina* a violet-blue pigment quite unlike that in *Velella* and *Fiona* in general appearance, distribution and chemical nature. But since it fed on *Velella* in much the same way that *Fiona* did, *Janthina* was also investigated in the manner described above. Feeding, with materials other than blue *Velella* tissue, had no effect on the abundance or intensity of the *Janthina* pigment. Nor did similar periods of starvation effect any noticeable change in the pigment. In brief, the pigment of *Janthina* is not related specifically to its food as seems to be the case with *Fiona.*

II

The determination of the chemical nature of the *Velella* pigment has been attempted several times. Lankester (1873) regarded it as probably identical with that of other Hydrozoa, but this view is no longer acceptable. A. and G. de Negri (1877) distinguished the pigment of *V. limbosa* spectroscopically from that of *Murex* and *Aplysia* by the absence of characteristic absorption bands. More recently Haurowitz and Waelsch (1926), in the course of a chemical analysis of the entire animal, examined the fresh animals spectroscopically and found diffuse absorption in the red and violet, but no sharp absorption bands.

The fresh tissues of *V. spirans*, when macerated in sea water and allowed to stand for four to six hours, yield a solution that closely approximates in intensity of color the blue of the living animal. The pigment may also be extracted in distilled water, though in this case only a portion of the contained pigment will appear in solution. These solutions, especially that in distilled water, show a reddish opalescence in reflected light. Near the neutral point it turns yellow, and successively pink and reddish-brown as it becomes increasingly acid. The pigment is not light-sensitive,—solutions kept in full or diffuse daylight for several days respond precisely as those kept in the dark. On exposure to air, the solution becomes acid with corresponding changes in color. On drying in air or in a dessicator, a reddish-brown, flaky, or amorphous crystalline mass results. This is soluble with difficulty in distilled water and does not yield the original blue color on increasing the pH to its value in the intact animal. Warming, or treating with alcohol, produces a flocculent white precipitate which is insoluble in water but soluble in alcohol and ether. The filtrate is red. Alkalis turn the solution blue-violet. Acids and boiling turn the solution red with the production of a flaky white or yellow coagulum, the latter being easily digested by pepsin. Excess of NaOH gives a violet color.

It was possible to make only some preliminary spectroscopic examinations of this pigment. The number of animals available was small, and it is necessary to use freshly prepared extracts, since under laboratory conditions they decompose rapidly by bacterial action. At 22° C. solutions retained their color for four days. Distilled water solutions showed diffuse absorption in the red,—$\lambda = 655\mu\mu$–$685\mu\mu$, and blue-violet $\lambda = 425\mu\mu$–$475\mu\mu$. The bands were not sharply demarked. This agrees with the findings of Haurowitz and Waelsch noted above. There was reason to suspect, of course, that the blue color of *Fionæ* which had been fed on *Velella* was chemically identical with the color of the latter. The blue dorsal papillæ of *Velella*-fed *Fionæ* were, therefore, extracted in the manner described above. These extracts, in both sea water and distilled water, reacted in precisely the same way and showed all the properties of *Velella* pigment solutions.

Several descriptions of animal pigments exist, none of which, however, corresponds in all details with the properties of the *Velella* and *Fiona* pigment. Apparently there is here present a protein combination upon which the blue color depends, but there is as yet no data as to its nature.

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THE SEX RATIO IN THE DOMESTIC FOWL IN RELATION TO ANTECEDENT EGG PRODUCTION AND INBREEDING

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A study of factors effecting a variation in the normal sex ratio of the domestic fowl should have an important bearing on the practical aspects of the poultry industry and on current theoretical questions concerning sex determination. During recent years considerable progress has been made in ascertaining the normal sex ratio of the domestic fowl, but this does not necessarily imply an increased ability to control sex. The term "sex determination" has reference to the causes which lead to the production of an individual of one or the other sex, whereas the term "sex control" is understood to imply that the causes which determine sex are more or less amenable to human control.

Factors that do not Affect the Sex Ratio

A number of observations have been made concerning factors which do not affect the sex ratio. That prenatal mortality does not affect the sex ratio has been determined by Thomsen (1911), Pearl (1917*a*), Crew and Huxley (1923), Jull (1924), Lambert and Knox (1926), Horn (1927), and Lambert and Curtis (1929).

That large eggs tend to produce male chicks and small eggs female chicks was the observation of Lienhart (1919), but the observations of Jull (1924) and Jull and Quinn (1924, 1925) show quite definitely that egg weight bears no relation to sex ratio. Jull and Quinn (1925) found no significant difference between the weights of male and female chicks at hatching time, and Jull and Heywang (1930) found that the percentage chick weight of initial egg weight is independent of the sex of the chick. Jull and Quinn (1924) also found that there is no relationship between the absolute length of the egg and the sex of the chick hatched from it nor between the relative length or shape of the egg and the sex of the chick hatched from it.

Jull (1924) showed that there is no relationship between yolk weight of egg and sex nor between yolk water content of egg and sex, and Jull and Heywang (1930) found that the percentage yolk weight of chick weight at hatching time is independent of the sex of the chick.

Lambert and Curtis (1929) and Christie and Wreidt (1930) observed no significant differences among sex ratios of families of hens of different ages.

ANTECEDENT EGG PRODUCTION AND THE SEX RATIO

The results secured by Pearl (1917*b*), Crew and Huxley (1923) and Jull (1922 and 1924) show that the greater the egg production prior to the hatching season the lower the sex ratio, although the results of Mussehl (1924), Lambert and Curtis (1929), Callenbach (1929), and Christie and Wreidt (1930) do not confirm these observations since they found no relationship between antecedent egg production and sex ratio.

The possibility of the sex ratio decreasing as antecedent egg production is increased is important from the practical standpoint because the continuous selection of high producing hens to be used as breeders each year should tend to give a high proportion of pullets. This is desirable from the economic standpoint since the major receipts in poultry husbandry are obtained from egg production. In order to secure further evidence on the relationship between antecedent egg production and sex ratio, the sex ratios were determined of families of ten or more chicks per family produced during the normal hatching season in 1925, 1926, 1927, and 1928 in flocks of Barred Plymouth Rock, Rhode Island Red, and White Leghorn yearling hens at the U. S. Animal Husbandry Experiment Farm, Beltsville, Maryland. The breeding females producing the families were divided into three classes of antecedent egg production, 0–29 eggs, 30–59 eggs, and 60–89 eggs laid prior to the hatching season from the beginning of the second year record in the case of each hen. The results are shown in Table I.

TABLE I

The Sex Ratio in Relation to Antecedent Egg Production in the Domestic Fowl

Breed	Antecedent Egg Production Classes		
	0–29 eggs	30–59 eggs	60–89 eggs
Barred Plymouth Rock	50.66±1.50	50.00±2.47	—
Rhode Island Red	51.76±0.67	51.96±1.04	52.35±3.37
White Leghorns	47.41±0.79	49.18±0.75	51.73±1.65

The data in Table I show that in the case of all three breeds there is no relationship between antecedent egg production and sex ratio. These results, therefore, are in conformity with those secured by

Mussehl (1924), Lambert and Curtis (1929), Callenbach (1929), and Christie and Wreidt (1930), and support the evidence tending to show that there is no relationship between sex ratio and antecedent egg production.

It is true that the observations of Jull (1924) were based on the annual egg production records of Barred Plymouth Rock pullets mated to Brown Leghorn cockerels, whereas the data given in Table I are based on yearling females mated to cockerels of the same breed. At the same time, it has already been observed that Lambert and Curtis (1929), and Christie and Wreidt (1930) observed no significant differences among sex ratios of the families of hens of different ages. Furthermore, Christie and Wreidt (1930) used a sex-linked back cross, F_1, males from a mating of a Black Minorca male with Barred Plymouth Rock females mated to Black Minorca pullets, and found no correlation between antecedent egg production and sex ratio, antecedent egg production being based upon the full year's production as in the case of Jull's (1924) work.

On the other hand, Christie and Wreidt (1930) observed an excess of barred progeny produced during February, March, and April as compared with the progeny produced during May, June, and July and an excess of black progeny produced during August, September, and October as compared with the progeny produced during May, June, and July. In the back-cross mating, heterozygous barred males on recessive black females, used by Christie and Wreidt, the progeny would normally be produced in the proportion of one barred male, one black male, one barred female and one black female. The excess of barred progeny produced in the early part of the season and an excess of black progeny produced during the latter part of the season is similar to the results secured by Jull (1924), but in Christie and Wreidt's case the sex ratio was not influenced. They offer the following suggestion, based on their results: "It is shown in this experiment that there is probably a seasonal effect of B (the sex-linked barring factor) and Jull's results may have been affected by this phenomenon." This matter is being investigated.

INBREEDING AND THE SEX RATIO

Callenbach (1929) secured results indicating that different strains of domestic fowl produce differing sex ratios. Mussehl (1924) secured results that show sex ratios from different males differing materially, and Jull (1924) secured sex ratios of 91.67 and 66.67, respectively, from two hens based on their total annual egg production. Christie and Wreidt (1930) observed that some of their females gave aberrant sex ratios, six each giving a low sex ratio and three each giving a high sex ratio.

Hays (1929) found that inbreeding tends to lower the sex ratio in Rhode Island Reds.

Moreover, Pearl (1924) observed that among matings of humans in Buenos Aires "there is some evidence of a significantly greater proportionate production of males in the offspring from matings involving different racial stocks than in the offspring from matings in which both parents belong to the same racial stock." Pearl analyzed the statistics of over 200,000 human births, extending over a period of ten years, among the following matings:

Argentine ♂ × Argentine ♀
Italian ♂ × Italian ♀
Spanish ♂ × Spanish ♀
Italian ♂ × Argentine ♀
Spanish ♂ × Argentine ♀

Conclusive evidence regarding the possible influence of inbreeding and outbreeding on the sex ratio among the various classes of domestic livestock is still very meagre but, as Pearl says, "Any data tending to throw light on the significance of any supposed sex-influencing factors can but be welcome." In the case of the domestic fowl it is a comparatively easy matter to secure relatively large families from single matings. Moreover, it is possible to practice close inbreeding over a period of years.

At the U. S. Animal Husbandry Experiment Farm, Beltsville, Maryland, matings involving crosses between breeds and varieties have been made and the sexes of the progeny recorded. Also, a number of pens of Barred Plymouth Rocks and White Leghorns have been inbred for a period of years and careful records kept of the sex of the progeny. The data from these matings are given here as a contribution to the general study of sex-influencing factors.

During 1927 a number of varieties of the Plymouth Rock breed were intercrossed as follows:

1. Buff Plymouth Rock ♂ × White Plymouth Rock ♀
2. White Plymouth Rock ♂ × Buff Plymouth Rock ♀
3. Silver Penciled Plymouth Rock ♂ × Partridge Plymouth Rock ♀
4. Partridge Plymouth Rock ♂ × Silver Penciled Plymouth Rock ♀
5. White Plymouth Rock ♂ × Barred Plymouth Rock ♀

In 1928 the progeny of each intercrossed pen were inbred, the amount of inbreeding being mostly half-sister-and-brother and in a few cases full-sister-and-brother matings.

In Table II are given the sex ratios of the families of the original crosses and of the families of the inbred matings made the following year.

The sex ratio of the families of the inbred matings is lower in three and higher in two cases than the sex ratio of the families of the original crosses, but in no case is there a significant difference.

TABLE II

Sex Ratios of Families of Plymouth Rock Variety Crosses and Subsequent Inbred Matings

Mating Number	Sex Ratio	
	Original Cross, 1927	Inbred Mating, 1928
1........	44.62±5.60	58.70±5.48
2........	58.23±5.12	46.36±4.19
3........	48.72±2.74	50.65±3.61
4........	48.70±3.95	44.86±1.21
5........	50.44±5.10	44.83±3.09
Total.......	49.77±2.02	47.32±1.62

In the fall of 1924 an inbreeding project was begun with each of four pens of Barred Plymouth Rock and White Leghorn pullets, each pullet being mated to an unrelated cockerel. Pedigree breeding was practiced so that the progeny for each male and female mating was definitely established. When the progeny of each pen reached maturity in the fall of the year, five full sisters and at least two of their full brothers were selected from the female breeder that had the largest number of progeny at the time of selection. Three full sisters were chosen from each of the two female breeders having the next highest number of progeny at the time of selection. The five full sisters and the two groups each of three half-sisters were mated to a full brother of the five full sisters. This was the method of selecting the breeders for each inbred pen in 1926, 1927, and 1928, the chicks raised in 1925 being the progeny of unrelated males mated to the original pens of females.

In Table III are given the sex-ratio data of the progeny of the original matings and of the progeny of the half-brother-and-sister and full-brother-and-sister matings the following year.

TABLE III

Sex Ratio of Families from Half-brother-and-sister and Full-brother-and-sister Matings

Mating	Sex Ratio
Original Barred Plymouth Rock mating	53.54 ± 1.89
Half-brother-and-sister Barred Plymouth Rock mating	50.21 ± 2.99
Full-brother-and-sister Barred Plymouth Rock mating	55.79 ± 4.46
Original White Leghorn mating	44.15 ± 1.33
Half-brother-and-sister White Leghorn mating	50.65 ± 1.82
Full-brother-and-sister White Leghorn mating	48.37 ± 2.52

The data in Tables II and III show that close inbreeding in the domestic fowl, at least for one generation, apparently has no significant influence on the sex ratio of the progeny.

In order to present further evidence bearing on the problem of the sex ratio in the domestic fowl in relation to inbreeding, the coefficient of inbreeding has been determined for the inbred families produced in 1926, 1927, and 1928 in the case of the inbred Barred Plymouth Rocks and White Leghorns.

Wright (1923) has pointed out that the coefficient of inbreeding (F) depends primarily on the number and closeness of the ancestral connections between the sire and dam, and secondarily on the degree of inbreeding of the common ancestors of the sire and the dam. "Every chain of generations in the pedigree by which one may trace back from the sire to a common ancestor and then forward to the dam, passing through no animal more than once (within the given chain), contributes to the inbreeding an amount equal to one-half used as a factor one more time than there are generations in the chain, with the qualification that this must be multiplied by a corrective term $(1 + F_A)$, in case the common ancestor (A) is himself inbred." Wright's formula for determining coefficients of inbreeding has been used in the analysis of the data discussed in this paper. "Let F_x and F_A be coefficients for the individual and for a representative common ancestor of his sire and dam, and letting n and n' be the number of generations between the sire and dam, respectively, and their common ancestor, we have as the general formula:

$$F_x = \Sigma\left[\left(\tfrac{1}{2}\right)^{n+n'+1}(1 + F_A)\right]."$$

It should be clearly understood that the coefficients of inbreeding given in this paper refer to the coefficients of inbreeding of the progeny of a given mating. For instance, when A is mated to his half-sister B, the coefficient of inbreeding of their progeny is 12.50 per cent. Also when C is mated to his full-sister D, their progeny have a coefficient of inbreeding of 25.00 per cent.

In Table IV the sex ratios of families representing different coefficients of inbreeding are given for both the inbred Barred Plymouth Rocks and White Leghorns.

TABLE IV

Sex Ratio of Families Representing Different Coefficients of Inbreeding

Coefficients of Inbreeding	Sex Ratios	
	Barred Plymouth Rocks	White Leghorns
12.50	50.68±3.99	48.82±2.92
25.00	43.90±3.45	46.63±2.00
37.50	38.57±5.93	44.20±4.83
50.00	—	41.94±7.41

The data in Table IV are consistent to the extent that in both breeds the sex ratio decreases as the coefficient of inbreeding increases. The sex ratios, however, do not differ significantly, as may be observed from the fact that in the Barred Plymouth Rocks the highest sex ratio is 50.68 ± 3.99 and the lowest is 38.57 ± 5.93, but the difference, 12.11 ± 7.14, is not significant. It is true that the observations are based on relatively small numbers of families and it is possible that observations based on larger numbers of families might show a significant relationship between inbreeding and the sex ratio.

Larger numbers of families are represented in the class of coefficients of inbreeding ranging from 21.51 to 29.50 and in the class ranging from 29.51 to 37.50 than in the coefficient of inbreeding classes given in Table IV. The sex ratios of the two larger family numbers of coefficient of inbreeding classes are given in Table V.

TABLE V

Sex Ratios of Families Representing Two Ranges of Coefficients of Inbreeding

Coefficients of Inbreeding	Sex Ratios	
	Barred Plymouth Rocks	White Leghorns
21.51–29.50	45.66±2.41	48.48±1.59
29.51–37.50	43.62±5.10	48.68±2.20

In the Barred Plymouth Rocks the class of higher coefficients of inbreeding has the lower sex ratio, but in the White Leghorns the reverse is the case. In neither case is the difference between the sex ratios significant. Moreover, the sex ratio of 48.68 ± 2.20 given in Table V for White Leghorns for the coefficients of inbreeding ranging from 29.51 to 37.50 is higher than the sex ratio of 46.63 ± 2.00 given in Table IV for White Leghorns for the coefficient of inbreeding of 25.00.

It seems apparent, therefore, that inbreeding in the domestic fowl can hardly be regarded as a sex-influencing factor, even when close inbreeding is practiced.

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THE EFFECT OF FATTY ACID BUFFER SYSTEMS ON THE APPARENT VISCOSITY OF THE *ARBACIA* EGG, WITH ESPECIAL REFERENCE TO THE QUESTION OF CELL PERMEABILITY TO IONS

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The subject of this study is the degree to which certain intracellular effects of acids may be modified by the presence of the salts of the acids. If a living cell is exposed to a penetrating acid and its salt, conditions being such that the salt is unable to penetrate, the internal reaction of the cell will be influenced by the absolute concentration of the acid rather than by the pH of the solution as such. Results which were interpreted in this way, as evidence of the relative impermeability of cells to salts, have been obtained for a variety of cells by Jacobs (1920*a*, 1920*b*, 1922*a*), Beerman (1924), Bodine (1925), and Lillie (1926, 1927). Furthermore, Osterhout (1925) and Osterhout and Dorcas (1926) have investigated the penetration of CO_2 and H_2S in a more quantitative manner on a particularly favorable material, *Valonia*, with results which led them to state that ". . . it is only the undissociated molecules which penetrate. . . . Under ordinary conditions there seems to be little or no exchange of ions."

On the other hand Smith and Clowes (1924*a*, *b*), and Smith (1925, 1926) found that certain physiological effects of the free acid in buffer systems of penetrating acids depended on the pH of the external solution, a result which they were inclined to interpret (1924*b*) as being due to the penetration of the salt of the acid rather than to effects on the external cell surface, since analogous external pH variations produced by non-penetrating acids were physiologically ineffective. Similar conclusions as to the penetration of certain salts have been reported by M. M. Brooks (1923) and by Haywood (1927).

The contradiction implied in the conclusions of these two groups of workers led to the present study of the question, in which the effect of acids on the apparent viscosity of the protoplasm of sea urchin eggs has been used as a measure of the intracellular effectiveness of the pH produced by various systems. The results obtained indicate that physiologically significant quantities of the salts of several penetrating acids enter the cells studied within a few minutes, in contrast to the ions of certain mineral acids, for which there was found no evidence of penetration under similar conditions. In this respect my work confirms the conclusions of Smith, but it has extended them to apply to considerably shorter times, and has led to a different conception of the manner in which the intracellular buffering action of the salt might be brought about.

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Material and Methods

The unfertilized eggs of the sea urchin, *Arbacia punctulata,* have been used throughout these experiments, since they are adaptable to semi-quantitative studies on the apparent viscosity of protoplasm. Because of the danger that the selective permeability of cells might be altered by injury, care was taken that the material should be as normal as possible. Eggs which were in good condition, as indicated by the prompt formation of fertilization membranes and by uniform cleavage (95 to 100 per cent), stood centrifuging without appreciable cytolysis and as a rule the controls maintained a uniform value of the apparent viscosity throughout an experiment. During the beginning and the end of the season, a sample of eggs of each lot studied was fertilized, and those lots selected for use which showed prompt and uniform elevation of fertilization membranes, but in the middle of the season such a selection was unnecessary. The occurrence of an apparent liquefaction on treatment with acid is not incompatible with subsequent cleavage and development after fertilization on return to sea water, although progressively less recovery is obtained as the degree of liquefaction is increased.

An experiment consisted of exposing the cells to the solutions in question, and determining the apparent viscosity of the protoplasm after various intervals. The method of centrifugation employed for these measurements was composed essentially of observations on the degree of movement of the yolk granules through the cell under the influence of a given centrifugal force acting for a constant time. Certain factors concerned in the resultant apparent viscosity of the protoplasm of these cells will be discussed elsewhere. For the present purposes such a determination of the rate of granule displacement has been used as a measure of the effects of acid, without attempting any analysis of the actual changes occurring in the apparent viscosity, or of whether changes occurred in the granules themselves. The term liquefaction is used in this paper to describe the effects studied, because it is probable from the results of various authors (Jacobs, 1922*b*; Edwards, 1923; and

Brinley, 1928) that at least a part of the physical effects of acid on the cell consist in the production of an actual liquefaction.

The granule displacement was evaluated in the following manner. After centrifuging, in order to prevent return redistribution of the granules in the cell, the eggs were immediately fixed in a solution of 0.04 per cent formaldehyde in sea water. The layers of material which are separated in sea urchin eggs by centrifugal force may be classified as a cap of fatty material, a hyalin segment of clear protoplasm, a segment of yolk granules, and finally the red pigment granules. Counts were made of the percentage of eggs showing a hyalin segment of an altitude of one-fifth or over of the diameter of the egg, out of a total number of 200 eggs which showed an axis of stratification perpendicular to the axis of observation. The position of the axis of stratification can be determined in a definite manner by locating the red granule layer opposite to the clear area. Since the estimation of the position of this axis is only possible in those eggs which show some granule displacement, the percentage actually counted is not an absolute percentage representative of the entire lot of eggs, although it tends to become so as its magnitude increases. The altitude of the hyalin segment was measured from the surface of the egg to the boundary between the hyalin and yolk segments, including within this segment the small amount of fatty material. The measurement consisted simply of a comparison with an ocular micrometer scale at a magnification of 100 diameters. The counts were reproducible with a probable error of plus or minus one per cent. The percentage of eggs showing this degree of stratification as determined in the manner described will hereafter be referred to as sigma.

Counts made on the same samples of eggs at intervals after the fixation showed an initial increase of about eight sigma during the first twenty minutes following fixation, after which the value remained constant for about two hours. A slow decrease in sigma of the order of 6 to 8 occurred after four to eight hours. Slightly lower concentrations of fixative did not give the initial rise but gave a more rapid drop, and higher concentrations tended to render the cells opaque. With the concentration of fixative employed, counts were made between one-half hour to two hours after fixation.

For values between 5 and 40, sigma increases nearly linearly with the time of centrifuging; but below 5 the curve of sigma against time is convex to the time axis, and above 40 to 60 sigma the curve tends to become concave. Treatment with fatty acids causes sigma to increase until a point is reached, with increasing concentrations of acid, where coagulation begins to occur, which is associated with an abnormal general appearance of the eggs. Coagulating concentrations of acid were not used in the present study of permeability.

Numerical values of sigma in different acid concentrations are of relative significance only, since considerable variations in absolute magnitude occurred among different lots of eggs. In the experiments described below, however, the relative effectiveness of a series of solutions was reproducible without exceptions. The effects described are based on a series of observations on three or more lots of eggs, for each series of solutions tested.

A centrifugal force of approximately 1000 times gravity was used, but this was not entirely constant from day to day, since the line current at Woods Hole fluctuates considerably. However, controls in sea water or unbuffered saline were included in each test, and with material in good condition sigma values were usually constant during each experiment, within the limits of error of the counts. The centrifuge was accelerated in a standard manner, twenty-five seconds being used to move the rheostat to its final point, and the machine established speed in about forty seconds after the current was turned on. Room temperatures varied between 22 and 25° C. during the course of the work, but the variations during any one experiment were not more than a few tenths of a degree. The cells were centrifuged for 2.0 to 2.5 minutes, the actual time suitable for each lot being determined in a preliminary test.

The technique of measuring the apparent viscosity differs from that previously employed by Heilbrunn (for numerous references see 1927) and by Barth (1929) in one fairly important respect. These workers, by centrifuging the material for various times, determined the shortest time required to produce granule displacement for comparison under different conditions. For the present purposes, I found that in cells centrifuged for any one time, with the technique used, the amount of granule displacement varied in different cells to such an extent that it was not possible to compare groups of cells centrifuged for different times unless comparisons were made on the basis of those times at which equivalent percentages of cells showed a given amount of granule displacement. In practice it was found more satisfactory to compare the percentages of cells showing a given amount of granule displacement after equal times of centrifuging.

Solutions.—Stock solutions of acids were made up by titration against standard NaOH, using phenolphthalein as indicator, dilution to the desired concentration, and retitration. The solutions of the salts of the fatty acids were made up by mixing 1.000 N NaOH and 1.000 N acid to a pH of 9.4 with thymol blue, this being the approximate pH of salt hydrolysis, allowing for salt error of the indicator, at salt concentrations of 0.5 molar. The salt solutions were kept on ice and made up fresh each week. Solutions for use on the eggs were made up daily from these stock solutions by volumetric dilution, with an accuracy of 0.5 per cent, in balanced isotonic saline media. For the first part of the work the saline medium was prepared daily from sea water, by treating it with 5 cc. of 0.5 N HCl per liter and passing a current of moist room air through it overnight. This procedure frees the solution from carbonates and CO_2 except for traces in equilibrium with room air. The solution was then filtered and neutralized with 0.25 N NaOH to an apparent pH of 7.2 with phenol red, which, corrected for salt error, becomes pH 7.0. To this medium were then added the acids to be tested on the eggs. However, for most of the work an artificial saline was prepared daily by mixing 1.00 M NaCl 420 cc., 0.50 M KCl 18 cc., 0.50 M $MgSO_4$ 51 cc., 0.50 M $MgCl_2$ 46.7 cc., and 0.50 M $CaCl_2$ 18.7 cc., and distilled water to one liter. These concentrations of salts were obtained from data on the relative salt concentrations of sea water, as given in the Tabulæ Biologicæ. The salinity was adjusted to the point where the medium caused no significant change in sigma, when compared with Woods Hole sea water. No difference in the results was observed between solutions made up in this saline and in the carbonate-free sea water.

When the volume of hypotonic reagents added to the saline exceeded one per cent, hypertonic NaCl was added in proper amount to restore the osmotic balance. Two per cent of distilled water in sea water was found not to affect sigma, while 10 per cent distinctly increased sigma. When the volume of the solutions added to the saline exceeded 10 per cent, the amounts of K, Ca, and Mg were adjusted to maintain the normal cation ratio, unless otherwise stated. A ten per cent variation in the cation ratio was not associated with appreciable variations in sigma. The effect of larger variations is described below.

All pH values given for HCl and phosphate solutions were determined with a quinhydrone electrode. The individual determinations were subject to potential drifts of one to three millivolts. The values given in Table I are averages of several determinations. During preliminary experiments, the pH of the solutions was found to remain unchanged during an experiment. The pH of solutions of fatty acid buffers were calculated from the known amounts of the free acid and its salt added, using constants of pK′ as 4.48 for acetic acid and 4.56 for valeric. The constant for acetic was taken from data of Michaelis and Krüger (1921) as the value of the constant in 0.5 M NaCl with 1 50 M $CaCl_2$, and the value for valeric was calculated from the accepted value for its pK, assuming that the effect of the salinity is the same as with acetic acid. For the second dissociation constant of phosphoric acid Michaelis and Krüger's value of pK′ in 0.5 M NaCl of 6.43 was used.

It will be noticed that the procedure followed in making up the experimental solutions differed from that used by Smith in his work on marine ova in one fairly important respect. Smith's method was to add standard sodium acetate to neutral carbonate-free sea water, and then to divide the resulting solutions into separate portions, and adjust the hydrogen ion concentrations to various values by the addition of HCl of the appropriate strength. This technique obviously lowers the salt concentration at the same time that it raises that of the free fatty acid, thus changing simultaneously the three variables: salt, acid, and pH. Under these circumstances the evaluation of the effect of each variable separately is difficult, and can be attempted only by a combination of a number of experiments. My procedure was so planned that each of the variables, acid, salt, and pH was in turn held constant while the other two were varied, thus rendering it possible to test Smith's conclusions directly in individual experiments.

The Effect of Certain Acids and Buffer Systems on the Apparent Viscosity

1. *The Effect of Mineral Acids on the Apparent Viscosity*

One of the arguments that ions in general do not penetrate living cells has been that cells are not injured or affected by the completely dissociated strong acids or bases in pH ranges in which the solutions of certain weak acids and bases, which are believed to contain a large proportion of undissociated molecules, can exert marked physiological effects apparently resulting from their ability to affect the hydrogen ion equilibria within the cell. The work of Bethe (1909), Warburg (1910), Harvey (1911), Jacobs (1920*b*), Chambers (1928) and others has shown that weak acids and bases produce intracellular indicator changes promptly under conditions where strong acids and bases are ineffective. A variety of physiological effects in animal, plant, and bacterial cells have been shown to follow the same rule in that, according to Loeb (1909), Cohen and Clark (1919), Jacobs (1924), Smith and Clowes (1924 *a* and *b*), Barth (1929) and others, they respond to weak organic acids more readily than to strong mineral acids, although in application the rule is not without its limiting conditions, especially when high concentrations of acids are employed (Crozier, 1916).

In the present experiments this general principle has been confirmed, in that HCl and the phosphate buffer system have been found to be ineffective in producing a liquefaction of protoplasm within the pH range in which the organic acids were effective.

Eggs were exposed to acids for various times, and they were then centrifuged and sigma values determined in the manner described above. The results of exposing eggs to HCl in unbuffered saline for fifteen minutes are given in Table I.

TABLE I

The Effect of HCl on the Apparent Viscosity

Experiment	Molarity	pH observed	Sigma
1	1.1×10^{-5}	—	15
	2.2	5.42	12
	4.3	5.08	16
	control	—	12
2	4.3	5.08	17
	control	—	17
3	5.4	4.41	12
	6.5	4.34	18.5
	8.7	4.28	15.5
	control	—	14.5

Since the variations of sigma in cells in HCl solutions are slight, and since there is no regular variation with the changes in acid concentration, it is considered that no significant effects on the apparent viscosity are produced by HCl at pH 4.3 or above. At about pH 4.3 the eggs tend to cytolyze, so that in view of complications due to cell injury, quantitative measurements of sigma below pH 4.3 were not undertaken. At pH 4.1 the protoplasm was not coagulated after fifteen minutes' exposure, and at pH 3.0 coagulation occurred.

Similar experiments were performed with phosphate buffers. Solutions of 0.00132 and 0.0066 molar NaH_2PO_4 in saline media were adjusted to a series of pH values between 4.2 and 5.8 with NaOH. No significant differences were observed between the apparent viscosity of cells in the saline media alone and in these phosphate solutions, after exposures of five to forty minutes. The ions of the phosphate system therefore appear to resemble the ions of HCl in being unable to influence the interior of the living *Arbacia* egg. The pH ranges used with these mineral acids are within those in which the fatty acids exert their effects; consequently the effects produced by the fatty acids cannot be due to the hydrogen ion concentrations as such which are produced in the external media.

2. *The Effect of Fatty Acids on the Apparent Viscosity*

The results obtained with the saturated fatty acids are very different from those just described for mineral acids. The effect of unbuffered valeric acid on protoplasmic viscosity is shown in Fig. 1. In this and all subsequent figures the ordinates represent the degree of liquefaction in sigma, and the abscissæ the time in minutes of exposure of the cells to the various solutions up to the time of centrifuging. In Fig. 1 it will be observed that with increasing time of exposure the degree of liquefaction increases up to a certain time, beyond which it tends to decrease. The magnitude of the maximum liquefaction and the rate of change increase with the concentration of the acid, between 1×10^{-4} and 3×10^{-6} molar.

Since the action of valeric acid may be due in part to the liberation of carbon dioxide from intracellular carbonates, a few observations on the effect of carbon dioxide were made. The gas was passed through sea water until a saturated solution was obtained. The latter was then diluted with sea water to certain pH values, determined with the quinhydrone electrode. A marked liquefaction was obtained at pH 5.1, and a coagulation at pH 4.9. These pH values correspond to carbon dioxide tensions of approximately 400 and 700 mm. respectively, according to the data of Henderson and Cohn (1916).

The magnitude of the liquefaction obtainable with unbuffered valeric acid is not very great. Cytolytic effects usually begin to occur above concentrations of 0.0001 molar, obscuring the possible effects of larger concentrations of acid on protoplasmic viscosity. Out of a total of seven experiments with unbuffered valeric acid, a liquefaction was observed six times; and in the lot of eggs in which no definite liquefaction could be observed, appreciable cytolysis was produced by 0.0001 N acid.

Using acetic acid in unbuffered solutions, I failed to observe a liquefaction in four experiments, using concentrations of 1×10^{-4} to 1×10^{-6} normal, although considerable cytolysis was observed in the higher concentrations. Consequently it is obvious that acetic acid produces intracellular effects less readily than valeric.

The effectiveness of different fatty acids was compared in buffered solutions, since under these conditions it was possible to obtain a definite liquefaction without cytolysis in all the acids used. The effect of the length of the carbon chain was studied with the acids acetic, propionic, butyric (a mixture of normal and iso forms), and *n*-valeric. Because of the almost equal strengths of the acids in question, such a comparison has a greater significance than would otherwise be the case. The results of a typical experiment with short exposures are shown in Fig. 2.

The concentration of the free acid was in each case 0.000517 N, in the presence of 0.000993 mols per liter of its salt. In this experiment the salt concentration was determined from the amount of standard NaOH added to the respective standardized acid solutions, instead of the salt having been made up separately as described above for the other experiments. The following pH values were calculated for these solutions, using the dissociation constants obtained by Drucker (1905) and assuming the correction of —.249 applied to pK for acetic acid to hold for each acid: acetate system 4.77, propionate 4.87, butyrate 4.81, and valerate 4.84. It is evident from Fig. 2, that the rapidity with which liquefaction is produced by such buffer mixtures increases with the increasing length of the carbon chain, irrespective of the slight differences in pH, which, except in the case of the propionic acid, would

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Plate I

Explanation of Figures

The figures show the effect of various acid solutions on the apparent viscosity of the *Arbacia* egg. The ordinates are the values of the viscosity in sigma, plotted in each case to the scale shown in Fig. 1, although only relative viscosities rather than absolute values are comparable in separate figures. The abscissæ are the times of exposure of the eggs to the solutions, up to the times of starting the centrifuging. The diagrams give the time in minutes, and it will be noticed that the time scales are not the same in all the figures. The curves are numbered in the order of increasing hydrogen ion concentration. The solutions as given were made up in balanced isotonic saline. The effects shown in Figs. 3 to 6 are typical of both acetic and valeric buffer systems.

Curve 1 in each figure represents the controls in sea water or in the unbuffered saline medium.

1. The effect of unbuffered solutions of valeric acid. Curve 2, valeric acid $2.9 \cdot 10^{-6}$ normal; curve 3, $1.74 \cdot 10^{-5}$ normal, curve 4, $1.16 \cdot 10^{-4}$ normal.

2. The effect of the length of the fatty acid carbon chain on the rapidity with which the liquefaction is produced. Curve *A*, acetic acid; curve *P*, propionic acid; curve *B*, butyric acid; curve *V*, valeric acid. The acid was in each case $5.17 \cdot 10^{-4}$ normal, in the presence of $9.93 \cdot 10^{-4}$ mols of the respective salt.

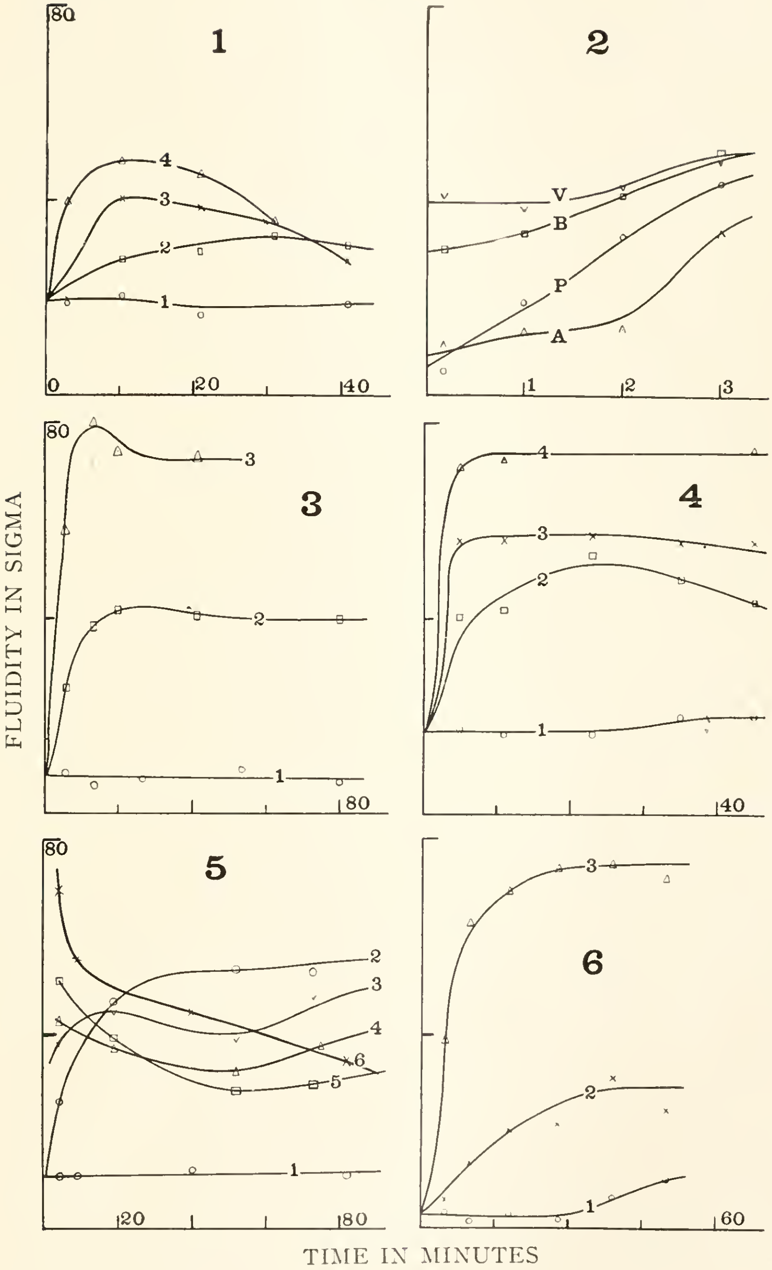
3. The effect of varying the pH by varying the concentration of the free acid in the presence of a constant amount of the salt of the acid. Curve 2, pH 5.2, free valeric acid 0.000341 normal; curve 3, pH 4.8, acid 0.000852 molar; sodium valerate in each case 0.00135 molar.

4. The effect of varying the pH by varying the concentration of the salt of the acid in the presence of a constant amount of free acid. Free valeric acid, 0.000585 normal. Curve 2, pH 5.6, sodium valerate 0.00585 molar; Curve 3, pH 5.0, valerate 0.00176 molar; curve 4, pH 4.6, valerate 0.000585 molar.

5. The effect of varying the salt of the acid in the presence of a constant amount of acid at a higher pH range than that of Fig. 4, namely 5.4 to 6.7. Free acetic acid 0.00096 normal, in the presence of sodium acetate of 0.008 to 0.16 molar. Curve 2, pH 6.7; curve 3, 6.4; curve 4, 6.0; curve 5, 5.7; and curve 6, 5.4.

6. The effect of increasing both the acid and the salt, while maintaining a constant pH of 5.17. Curve 2, free valeric acid 0.00029, valerate 0.00117; curve 3, acid 0.000870, valerate 0.00351 molar.

Plate I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FLUIDITY IN SIGMA
TIME IN MINUTES

be expected to reduce rather than to enhance the observed effects of the permeability differences.

After three to five minutes' exposure to the fatty acid solutions, there is shown a tendency of the viscosity values to reach the same equilibrium point. However, an identical equilibrium is not realized, and even after twenty minutes' exposure the acetic acid has usually produced somewhat less liquefaction than the valeric, in spite of the fact that it is a slightly stronger acid, this behavior recalling the comparative ineffectiveness of acetic acid in unbuffered solutions.

The observation that the rate of production of the protoplasmic liquefaction increases with the length of the fatty acid carbon chain accords with the general principle of Overton, which seems to hold for a great variety of material (Jacobs, 1924), that in homologous series, the more highly the non-polar portion of a molecule is developed as compared with the polar, the more readily are intracellular effects produced. The ineffectiveness of the mineral acids in this case cannot be due to their low concentrations, for HCl fails to produce a liquefaction at a concentration of 8.7×10^{-5}, while valeric acid is effective at 3×10^{-6} N. Furthermore, if we consider the buffering capacity of the solutions used ($\Delta B/\Delta pH$, Van Slyke, 1922) over the pH interval of 4.77 to 6.80, the latter taken as the normal pH of the cell contents (as determined for other *Echinoderm* ova by Chambers, 1928), it is seen that the effective acetate solution has a buffering capacity of only 0.00025, while for the ineffective phosphate buffer it is 0.0023.

3. *Penetration of the Cell by the Salts of Fatty Acids*

So far the results have not been in disagreement with the conceptions that ions penetrate uninjured cells only with considerable difficulty, if at all. If now we consider the salts of the fatty acids, which are believed to be completely ionized, we obtain quite a different picture. For the following experiments in this section both acetate and valerate buffers have been used.

(a) *The Effect of the Salt of the Fatty Acid on the Apparent Viscosity*

As a preliminary to the study of the effect of the acid in the presence of the salt, observations were made on the effect of the salt alone. Sodium acetate in the saline media without the presence of free acid, other than the traces resulting from hydrolysis, was found to produce no significant changes in sigma in concentrations of 0.001 to 0.004 molar. In solutions of 0.008 and 0.01 molar, a liquefaction was observed after twenty minutes' exposure which became more marked after longer exposures. The effect was variable in degree, but the liquefaction was at times as great as thirty sigma above the controls after forty minutes' exposure to 0.01 molar sodium acetate. In the presence of a potassium excess of 0.01 molar, the acetate effect was increased, *i.e.*, it was manifested sooner and in greater degree. Lithium acetate, 0.01 molar, produced an even greater effect than potassium acetate. The lithium and extra potassium, when added in the form of chlorides to the standard saline, caused no significant changes in sigma; therefore, this liquefying effect of lithium or of excess potassium appears to be associated with the presence of the acetate ion.

(b) *The Effect on the Apparent Viscosity of Changing the pH by Varying the Free Acid in the Presence of Constant Salt*

In a concentration range in which the salt alone produced no detectable effects, it may be shown that the effect of the acid on the apparent viscosity in the presence of the salt is similar to the effect of the acid in the absence of the salt in the case of valeric acid described above. The result of varying the free acid in the presence of a constant amount of salt is shown in Fig. 3. It is seen that, in the presence of the salt, as with the acid alone, increasing degrees of liquefaction are associated with increasing concentrations of free acid. This result would be expected whether or not the salt penetrated. The concentrations of acid used are greater than those possible in unbuffered solutions without gross injury; and the liquefaction produced is also greater and considerably more prolonged.

(c) *The Effect on the Apparent Viscosity of Changing the pH by Varying the Salt while the Free Acid is Constant*

If the salt penetrates readily, it would be expected that altering the pH by varying the salt concentration in the presence of a constant amount of free acid would result in immediate differences in the degree of liquefaction produced by the acid. That such is the case is shown in Fig. 4, in which the effect of valeric acid 0.000585 N is shown to be modified by sodium valerate of 0.00585 to 0.000585 molar. It is seen in Fig. 4 that the degree of liquefaction increases with the increasing hydrogen ion concentration and the decreasing amounts of salt. At pH 4.6 to 5.6 this effect is marked in both the valeric and the acetic solutions. The effect of the salt appears within four minutes of exposure to the solutions, the shortest time tested. It is presumably not a specific salt effect, since it occurs in salt concentrations of 0.002 to 0.0006 molar, which are below those where the salt effect was observed in solutions of sodium acetate without added free acid, and also because the liquefaction increases rather than decreases with the decreasing salt concentration. It is probably not caused by an influence of the salt on the rate of penetration of the acid, since the acid penetrates very quickly and apparently establishes a distribution equilibrium within three to eight minutes in solutions of pH 5.2 or below.

It seems most probable that the influence of the salt is due to an actual buffering effect exerted intracellularly. If this is so, these experiments indicate that the effect of the acid on the apparent viscosity is exerted through some influence on the intracellular hydrogen ion equilibria, rather than through a more specific molecular reaction, since, when the total acid concentration is constant, variations in the hydrogen ion concentration alter the effectiveness of the acid. It is believed, therefore, that these experiments constitute evidence that the salts of fatty acids are able to penetrate the living cell. In regard to the comparative rates of penetration of the acids and their salts, it is shown in Fig. 2 that acetic acid does not exert its characteristic effect until after about three minutes' exposure. Since the buffering action of sodium acetate may be in evidence after four minutes' exposure (the shortest time tested), it appears quite possible that the salt penetrates the cell, in the presence of the acid, at a rate which is of the same order of magnitude as that of the acid.

When a similar experiment is performed in which the free acid is kept constant and the salt varied, within a higher pH range, *i.e.*, between 5.7 and 6.7, somewhat different results are obtained. In order to produce a measurable liquefaction at these pH values, both the acid and the salt must be increased to within the range of salt concentrations at which the salt produces specific effects on the apparent viscosity. The results of such an experiment are shown in Fig. 5. The free acid in this experiment was 9.6×10^{-4} N, and the salt concentrations were up to 0.16 molar. The pH values calculated were, for curve 2, 6.7; curve 3, 6.4; curve 4, 6.0; for curve 5, 5.7; and curve 6, 5.4. It is seen that the initial effect is a liquefaction which increases with the hydrogen ion concentration, as was observed for the lower pH ranges described above. However, with increasing exposures, sigma values decrease in the solutions of lower pH and rise in the higher, with the result that, after an hour or more of exposure, between pH 5.7 and 6.7 the order of increasing fluidity has become reversed and consequently the liquefaction increases with the salt concentration instead of with the external hydrogen ion concentration.

Since the order of increasing fluidity may be reversed in this way, it is possible that the liquefying action of the salt may be of a different character from that of the acid. In view of the many factors which may affect the apparent viscosity, it is not unlikely that the seat of action of the hydrogen ion may differ from that of the salt. In this connection, the possibility was considered that the influence of the salt on the apparent viscosity was the result of an osmotic effect. The solutions of sodium acetate in saline media were made up to be isosmotic with normal saline, but if the salt should penetrate and remain osmotically active, these solutions would not be isotonic for the cell; in fact, the solutions used in the experiment shown in Fig. 5 would in effect be of various degrees of hypotonicity down to 68 per cent of the isotonic strength, and the expected changes in volume would result in an increased apparent liquefaction such as was observed. That this is probably not the case, however, is indicated by the fact that measurements of cell volumes in such solutions, kindly made for me by Miss D. R. Stewart by a technique which she will describe elsewhere,[2] revealed no significant changes. In this respect, then, the sodium salts of the fatty acids differ from the ammonium salts, which Stewart (1929) has found to cause osmotic swelling. These results may be due to the fact that the sodium salts can enter only in osmotically negligible amounts, but, on the other hand, it may be that their entrance is for some other reason not associated with an increase in the total osmotic pressure of the cell.

(d) *The Effect on the Apparent Viscosity of Increasing the Total Acid at Constant pH*

When the pH is kept constant and the free acid and the salt increased simultaneously a greater liquefaction is produced as the free acid concentration becomes larger, as shown in Fig. 6. Indeed, a coagulation was obtained with free acid above 0.001 N at pH 5.2. This result might be due to the fact that relatively less salt than acid penetrates the cell, so that the intracellular hydrogen ion concentration would become greater with the larger amount of acid. On the other hand there is an alternative possibility that the buffering capacity of the penetrating acid solution is one of the factors concerned in the magnitude of the changes produced. That buffering capacity is of importance in these experiments may be inferred from the work of Chambers (1928), Reznikoff and Pollack (1928) and Pollack (1928), who find that various cells have considerable buffering power, sufficient indeed to prevent changes in the colorimetrically determined pH of the hyalin protoplasm, within the observational limits of ± 0.1 pH, when treated with sublethal concentrations of CO_2. Hence it would be expected that

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[2] See Stewart, *Biol. Bull,* in press.

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the production of an acid liquefaction in the present experiments is not associated with an equalization of the intracellular and environmental pH, but only with a certain shift of the pH of the cell. The degree of this shift would be a function not only of the pH itself, but also of the buffering capacity of the penetrating components of the medium. Consequently, on this basis, one would expect the results obtained in this experiment, and such effects cannot be considered as evidence that the salt penetrates less readily than the acid.

A POSSIBLE MECHANISM BY WHICH THE SALTS OF PENETRATING ACIDS MIGHT ENTER THE CELL

The apparent permeability of the cell to the salts of the penetrating fatty acids presents a rather surprising contrast to its relative impermeability to certain other ionized compounds. This situation might be due to specific differences in the behavior of the different ions toward the cell membrane, but there is no independent evidence that the physical properties of the salts of the various acids are related to the physical properties of the acid molecules to an extent which would account for these physiological peculiarities.

An alternative explanation may be developed from the following considerations. In general, ionized compounds do not penetrate cells at all freely, but it is possible that the restrictions to the passage of ions in many cases are imposed chiefly on ions of one sign. The erythrocyte, for example, under ordinary physiological conditions, appears to be a cell which is permeable to anions and impermeable to cations (for references see Warburg, 1922 and Van Slyke, Wu, and McLean, 1923). The fact that the erythrocyte is freely permeable to ammonium chloride and similar ammonium salts has been explained by Jacobs (1927) as being due to the free penetration of the cell by NH_3 and subsequent exchange of the internal OH ions for the external anions of the salt. With the majority of cells, it is possible that the conditions prevailing in the erythrocyte may be reversed, such cells under ordinary conditions being more permeable to cations than to anions. The evidence for other cells is less direct than in the case of the erythrocyte, and is chiefly potentiometric, the sign or magnitude of the potentials measured across membranes responding to changes in the concentration or character of the salt present in the manner which would be expected if the anions could not pass through the membrane while the cations tended to diffuse. The application of the principles involved has been discussed by Michaelis and others (1925, 1927, etc.). Potentiometric evidence of this nature has been reported by Loeb and Beutner (1911) and by Fujita (1925) for the apple skin, by Mond (1927) for the wall of the stomach, by Amberson and Klein, (1928) for the frog skin, and by Sumwalt (1929) for the chorion of the *Fundulus* egg. Michaelis and Fujita (1925) have furnished in addition chemical evidence of the permeability to cations and the impermeability to anions of the apple skin. It is generally known that a normal surface is positive to an injured area in nerve, muscle, and apple, and this sign of the demarcation potential is not incompatible with a greater permeability of the normal surface to cations than to anions. Furthermore, Mond and Amson, (1928), from analyses of perfusing fluids, concluded that frog muscle is permeable to certain cations, *e.g.,* $K^{\cdot}$ and $Cs^{\cdot}$, but is impermeable to chlorides.

If the *Arbacia* egg is permeable to certain cations, its apparent permeability to the salts of penetrating acids might result from the entrance of the cell by the organic acid in the undissociated form followed by a transfer of cations, the external base being exchanged for the hydrogen ion derived from the intracellular dissociation of the acid. This process would result in the presence of acid anions and additional base inside the cell without an actual transfer of anions across the membrane. There is evidently an analogy between this mechanism and that of the penetration of the anion-permeable erythrocyte by a salt whose cation enters in an indirect manner. However, conditions are not completely analogous, since the penetration of the erythrocyte by ammonium chloride causes marked osmotic swelling, whereas this does not appear to be the case in the penetration of the *Arbacia* egg by potassium acetate.

If the transfer of base takes place by an exchange of alkali cations from without for hydrogen ions from within, the mobility of the cations in passing through the membrane might be the limiting factor in the rate at which the intracellular buffering action of the salt becomes manifest. Michaelis and collaborators (1925–1927) have observed that in the dried collodion membrane the mobility differences of the cations are greatly exaggerated. Netter (1928), Brooks (1930), and others have applied these considerations to explain the accumulation of K by many cells as a result of a cation exchange of hydrogen ions, produced metabolically, for K ions from the outside rather than for Na ions, since, although the latter ions are present in much the greater concentration in the environment, their diffusion may largely be restrained by a membrane which permits the passage of K ions. The presence of a membrane of this type in *Arbacia* eggs is suggested by the high ratio of K to Na found in the cells in comparison with that in sea water. Blanchard (unpublished results) reports a ratio of equivalents of K to Na of 1.90 in *Arbacia* eggs, while in sea water the ratio is 0.0213.

If this property of the cells results from characteristics of the membrane, it would be expected that, in the exposure of the cells to an acetate buffer in the balanced saline media, the base transferred would be K rather than Na, and that consequently changes in the K concentration of the medium would affect the rate of entrance of the base, and thereby the degree to which a given amount of salt is able to exert an intracellular buffering action after short exposures. This was observed to be the case under the following conditions. Certain amounts of 0.05 N NaCl, KCl, or RbCl were added to solutions of acetate buffers, with free acetic acid 0.00059 N at pH 5.0, in the usual saline media. As already mentioned, variations in K concentration of this magnitude in the absence of acetates produced no significant changes in sigma. The effects on the degree of liquefaction produced during the first two to nine minutes of exposure to such solutions are shown in Table II. The results are given in sigma reduced to a common basis by taking the values obtained in the high K concentration as twenty sigma.

TABLE II

The Effect of Various Cations on the Liquefaction Produced, by an Acetate Buffer

Concentration of chlorides added *mols/liter*	Rb	K	Na
0.002	—	20	35
0.002	7.5	20	27.5
0.01	14	20	28
0.004	13.5	20	—
0.003	—	20	32

In these experiments the absolute differences are small, but the relative effects of the cations always appeared in the order given at this pH. At higher pH values, where the salt reversal of the pH effect occurred, the cation series was in most cases reversed also. However, if we consider only the pH range where the salt appears to exert a buffering effect which is not complicated by other factors, the degree of initial liquefaction produced is in the order Rb<K<Na. After longer exposures, there is a tendency for the same equilibrium point to be reached, but this is not maintained for over twenty minutes in some cases. It will be noted that the series obtained is compatible with the view that the degree to which the salt is able to buffer the acid intracellularly during the first few minutes of exposure, and therefore the rate of penetration of the salt, increases with the amount of the more highly mobile cations present. Although physical properties

other than mobility may be concerned, nevertheless the effect is suggestive of an actual penetration of base rather than a removal of acid as suggested by Smith.

When the amounts of acetate in the medium are as high as 0.1 molar, such a cation exchange would probably result in an abnormally great accumulation of K in the cell relative to Ca. Chambers and Reznikoff (1926) have observed that the injection of NaCl or KCl into cells causes a liquefaction, whereas Ca causes a coagulation. This observation suggests that the liquefaction produced by the acetate salt may be due, in part at least, to the accumulation of K.

Discussion

Evidence has been presented that the salts of penetrating acids are able to enter sea urchin eggs within four minutes or less, although certain other ionized compounds are apparently not able to penetrate these cells. The evidence is based on the observation that the salt of a penetrating acid alters the degree to which the acid is able to affect the apparent viscosity of the protoplasm, an effect presumably exerted by virtue of the influence of the acid-salt mixture on the intracellular hydrogen ion equilibria.

This evidence of the penetration of cells by the salts of penetrating acids supports the conclusions arrived at by Smith with other methods on marine ova and cardiac muscle, and by Haywood on skeletal muscle, so that this type of permeability seems to be found in a certain variety of cells. These findings differ from those of Jacobs, Lillie, Osterhout, and others on a number of other cells, and it seems reasonable to suppose that the different results may be due to rather considerable differences in the relative rates of penetration of certain acids and their salts into the various types of cells in question. Since, however, it was noted by Jacobs that the differential effects produced by CO_2–bicarbonate mixtures on *Symphytum* and on starfish eggs tended gradually to disappear, the difference is probably only a quantitative one. This view is substantiated further by the observation of M. M. Brooks (1923) that bicarbonate penetrates into *Valonia* after eighty minutes' exposure. Lillie's observations on starfish eggs were limited to exposures of five to fifteen minutes, and under these conditions the pH of the medium usually seemed to be a less important factor physiologically than the changes in the concentration of acid available for diffusion into the cell; however, the experiments do not exclude the possibility of the penetration of salt.

The present observations of the effects of acids on protoplasmic viscosity have been discussed in terms of permeability. It has been shown by other authors that many organic acids diffuse through certain living tissues where mineral acids do not. Since organic acids applied externally cause color changes in granules stained by indicators in marine ova although mineral acids do not have this effect, it is probable that the fatty acids actually penetrate. If fatty acid enters marine ova, it is assumed, until proven otherwise, that the salt penetrates and buffers the acid directly in the cell, rather than that it acts from a distance in some unknown manner. Strictly, the term penetration has only the justification of being a convenient description of the selective physiological reactivity with which the present study is concerned.

Summary

The effects of the pH of the medium on the apparent viscosity of *Arbacia* eggs have been studied by means of an adaptation of the centrifuge method.

It was found that the degree to which fatty acids decrease the protoplasmic viscosity can be altered by the presence of the salt of the acid, apparently by virtue of the influence of the salt on the intracellular hydrogen ion equilibria. Similar pH variations of the medium produced by mineral acids do not affect viscosity.

These results offer confirmation, from a separate type of evidence, of Smith's observation that the salts of penetrating acids differ from other ionized compounds in apparently being able to penetrate the living cell much more easily. It is suggested that cell permeability to this type of salt could be explained as resulting from a cation exchange of external base for internal hydrogen ions from the penetrating acid, the entrance of the salt therefore being accomplished without the direct transfer of its anions.

It is a pleasure to acknowledge my indebtedness to Dr. M. H. Jacobs for the suggestion of this problem, advice during its development, and careful criticism of the manuscript.

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THE PERMEABILITY OF THE *ARBACIA* EGG TO NON-ELECTROLYTES

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I

The most extensive systematic studies of the permeability of cells to dissolved substances have been made upon plant material. Particularly noteworthy in this connection is the work of Overton (1895, 1907) and of Bärlund (1929) (see also Collander and Bärlund, 1926). Among animal cells the mammalian erythrocyte has proved to be especially useful and has been most frequently employed (Gryns, 1896; Hedin, 1897; Hamburger, 1902; Mond and Hoffman, 1928; Fleischman, 1928, and others). Because of the highly specialized nature of this latter type of cell, however, it is desirable, before using the results obtained with it as a basis for extensive generalizations, to supplement them with similar observations upon a variety of other animal cells. The present paper deals with experiments upon the relative rates of penetration of nineteen different non-electrolytes into a cell of animal origin whose permeability to dissolved substances has not hitherto been systematically studied, namely, the egg of the sea urchin, *Arbacia punctulata.*

Among the peculiarities which make the *Arbacia* egg a suitable form of material for such investigations are its spherical shape, which permits a study of permeability by direct measurements of volume changes, its convenient and almost uniform size, its even and not too rapid rate of swelling, and the large numbers in which it may be obtained from a single female. Its chief disadvantage lies in the amount of inert yolk material which it contains. This cell was first employed for permeability studies by Lillie (1916), who compared the rates, before and after fertilization, at which water entered the egg from anisotonic solutions. Further extensive work upon its permeability to water, under a variety of conditions, has been done by McCutcheon and Lucké (1926 and subsequent papers in the same journal).

Though the present investigation is concerned primarily with the penetration of dissolved substances rather than of water, the actual nature of the observations is the same in the two cases, consisting in measurements of the progressive changes in the diameter of the eggs during the course of the experiment. In the work of the previous investigators the movement of water, and the volume changes incident to this movement, were determined solely by the initial osmotic differences between the eggs and the surrounding solution. In these experiments there are, in addition, such further osmotic inequalities as are set up by the diffusion of the dissolved substance into the eggs.

According to the experimental procedure employed, the volume changes accompanying penetration of a solute may be of two types. The first involves swelling in a pure solution of a penetrating substance and should theoretically continue indefinitely, but actually results in the eventual cytolysis of the egg. The second occurs in solutions containing both penetrating and non-penetrating substances; in it, following a preliminary shrinkage, the swelling continues until the cells have attained an equilibrium volume determined by the concentration of non-penetrating substances in the solution.

In Fig. 6*A* are represented typical curves of the former type, obtained by exposing eggs to solutions of ethyl and methyl alcohols approximately isosmotic with sea water. They illustrate the rapid and continued swelling of the eggs in a pure solution of a readily penetrating substance. Figure 6*B*, for the same substances dissolved in sea water, represents the other type of volume change which occurs in solutions that are initially hypertonic, due to the addition of some non-penetrating substance. Both types of procedure have been used in the present experiments, but the second has been found to be more generally satisfactory.

The actual technique used in handling the eggs in these experiments was essentially that described by McCutcheon and Lucké (1926). Eggs from a single female were obtained with due care to avoid contamination by either sperm or intestinal debris. They were rinsed several times in filtered sea water and allowed to stand, without crowding, in dishes set in running sea water. From these they were removed, as needed, by means of a capillary pipette. Ordinarily about 100 eggs, in the smallest possible quantity of sea water, were used for each experiment. All measurements were made at room temperature, which varied from 21° to 25° C. during the summer, but not over a degree on any given day. The temperature of the running sea water in which the eggs were set averaged slightly lower, varying from 20.5° to 23° C.

In each experiment a watch-glass containing 7 to 8 cc. of the solution to be tested was placed on the mechanical stage of a microscope, the eggs introduced by means of a capillary pipette, and the water immersion lens quickly focussed. A group of five spherical eggs was selected and their diameters recorded by means of a filar micrometer. The total magnification afforded by the system used was 188 diameters. The eggs in each group were always measured in the same order and at as nearly equal time intervals as possible. Eggs which were not changing markedly in size could be measured with an error of no more than 0.7 per cent of their total diameters, and even if readings were taken at maximum speed on rapidly swelling eggs, the error was not greater than twice this amount.

The necessary pH determinations were made colorimetrically, using phenol red and bromocresol purple as indicators. It was not always possible to keep the reaction of a given solution constant during an experiment, but readings were taken at the beginning and end of each series and the results were discarded if the variation was found to be too great. The eggs do not seem to be osmotically sensitive to small changes in pH, as such, in regions not far removed from neutrality, so that a variation of about 0.2 of a pH unit in half an hour was considered allowable. No correction was made for the salt error of the indicators, since it was merely desired that solutions which were to be compared should be made up to reproducible pH values.

The experimental results have been presented in the form of curves, plotting diameter against time. Each point on a curve represents the average diameter of the five eggs under observation at that time. All the curves in any single figure were obtained on the same day and with eggs from the same female. Since volume is a function of diameter, the latter was used in plotting the curves in order to avoid laborious calculations. Actual figures for the volumes were obtained, however, for the preparation of Table I.

Since the eggs deteriorate on standing, only a limited number of experiments can be done on those from any single female. On the other hand, different lots of eggs vary enough in their size and permeability so that results on material from different animals cannot fairly be compared. As a result of these difficulties, the relative rates of penetration of a long series of compounds can be arrived at only by piecing together the results of a number of shorter, overlapping series.

Even in the same lot of eggs it is desirable, where accurate comparisons are to be made, that the particular group selected for measurement in each solution shall agree in average initial size and final equilibrium volume. Since the eggs in each set must be selected at random in a very short time, and averages cannot be obtained until the end of the experiment, it frequently happens that the observations must be repeated several times before an unobjectionable comparison is possible. The discarded results are not a total loss, however, since, while they are not in themselves conclusive, they furnish a certain amount of confirmatory evidence. A considerable number of experiments of this type have been performed but are not reported in this paper, though their results are in general agreement with those actually cited.

The best method of presenting the experimental data would obviously be by the use of complete swelling curves. Since it is impossible, however, to reproduce here more than a few of the curves that have been obtained, it has seemed best to make comparisons of the times required in different solutions for the eggs to reach some arbitrary degree of swelling. A convenient volume increase for most substances is that represented by a size halfway between the initial and equilibrium volumes; for more slowly penetrating substances 5 per cent or 20 per cent increases have sometimes been employed. While this method of presenting the results admittedly leaves something to be desired, it should be remembered that in every case the entire curve has been plotted and used as a basis for the conclusions drawn with regard to the behavior of the various substances.

II

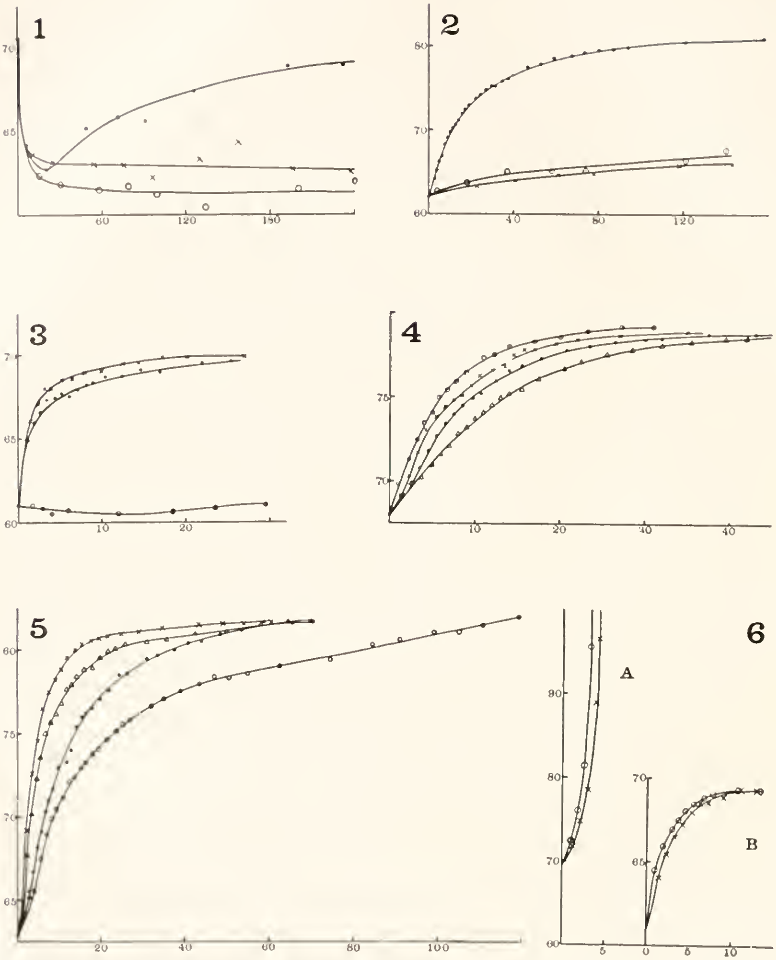
The results of the most conclusive experiments on the relative rates of penetration of different substances are presented in tabular form in Tables I, II, III and IV. Additional features of a few of the experiments are shown graphically in Figs. 1 to 6, inclusive. In the following more detailed description of these results the various substances studied are grouped according to their chemical relationships.

1. Water

(50 experiments.) Experiments involving the entrance of water alone were run frequently to serve as controls for those with other substances. In Fig. 4 and in Tables I, II and III are given the results of a number of such experiments. They showed comparatively slight variation from day to day during two successive summers, the time for swelling halfway to equilibrium in 60 per cent sea water averaging very close to four minutes, with maximum variations of from 2.6 to 5.8 minutes. These figures agree well with those obtained by McCutcheon and Lucké (1926) under similar experimental conditions.

2. Monohydric Alcohols

The two monohydric alcohols that were tested both entered *Arbacia* eggs with great ease. Cells placed in molar solutions of methyl and ethyl alcohols in distilled water doubled their volumes in three or four minutes (Fig. 6). If the alcohols were dissolved in sea water, swelling



FIGS. 1–6: The swelling of *Arbacia* eggs in various solutions. The abscissæ are times in minutes; the ordinates are diameters in arbitrary units, each of which is equal to 1.064μ. Each point represents the average diameter of five eggs except in a few instances when ten eggs were measured. In all the experiments except 1 and 6.*A* there was a ten-minute preliminary shrinkage of the eggs in a solution of glycerol made up in the same way as the solution to be tested. pH 8.2

1. Glycerol (●), erythritol (×) and dextrose (○); molar solutions made up in 60 per cent sea water. Fertilized eggs, ten eggs measured for each point.

2. Ethylene glycol (●), urea (○) and glycerol (×); molar solutions in 60 per cent sea water.

3. Monacetin (●), diacetin (×) and glycerol (○); molar solutions in sea water. Solutions brought to approximately pH 7.0.

4. Butyramide (/), propionamide (●) and acetamide (△), made up to half molar concentration in 60 per cent sea water; (○) 60 per cent sea water alone. The two breaks in the butyramide curve correspond to times when abnormalities

was also rapid, being faster than for glycerol, urea, ethylene glycol, acetamide or formamide, and at about the same rate as that for monacetin or for water alone. Some of the more toxic compounds such as ethylene glycol mon- and diacetates, diacetin and monochlorhydrin seemed to show a more rapid penetration, but this was perhaps due to injury effects on the eggs.

Methyl Alcohol (16 experiments) regardless of solvent, invariably entered the eggs more slowly than did ethyl alcohol, though the differences were slight (Fig. 6). In molar solutions in distilled water the average of the experiments gave, for the times necessary for a 50 per cent increase in volume, 2.9 minutes for ethyl and 3.9 minutes for methyl alcohol (Table IV). Another experiment in which molar solutions in distilled water were used gave values of 4.1 and 3.2 minutes, respectively (Table I).

Ethyl Alcohol (10 experiments), as just mentioned, gave a rate of swelling slightly greater than that for methyl alcohol. Sometimes this rate appeared to be even faster than that for water alone; in any case, it was not significantly slower.

3. Di- and Polyhydric Alcohols

Three alcohols belonging to this group were studied and were found to be slower in their rates of penetration than the monohydric alcohols; they covered the range from moderately rapid entrance to apparently none at all, as the size of the molecule and number of polar OH groups were increased.

Ethylene Glycol (70 experiments) is the most satisfactory to work with of all the compounds tested, since it seems to be entirely non-toxic to the eggs when dissolved in sea water and only very slightly so in other solvents. It has been used in solution in sea water, 60 per cent sea water, M/3 $CaCl_2$, M/2 KCl, M/2 NaCl and distilled water. Its rate of penetration is intermediate between that of substances such as glycerol or urea, which enter very slowly, and those of the large group of rapidly penetrating substances of which ethyl alcohol and diacetin are typical. In solutions made up in 60 per cent sea water approximate equilibrium is attained in about two hours, a period long enough so that the swelling process can be studied in detail. The rate of penetration from sea water

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occurring in the original eggs necessitated shifting the observations to different individuals.

5. Fertilized (●) and unfertilized (○) eggs in ethylene glycol made up to molar concentration in 60 per cent sea water; fertilized (×) and unfertilized (△) eggs in 60 per cent sea water alone.

6. Ethyl (○) and methyl (×) alcohols. *A,* molar solutions pH 7.0; *B,* molar solutions in sea water.

solutions has been compared with that of each of the other substances investigated. Of the results obtained, two sets which permit comparisons with the very slowly penetrating urea and glycerol (Fig 2) and with water (Fig. 5) have been reproduced in graphic form.

Because of their almost identical molecular weights, it is interesting that the rates for urea and ethylene glycol should be very different, a 20 per cent increase in volume in one experiment (Table I) requiring 4.8 minutes for ethylene glycol and 100 minutes for urea. In another experiment a 5 per cent increase in volume occurred in about two minutes in the one case and in thirty-six minutes in the other. A number of additional experiments are included in Tables I, II, III and IV, covering comparisons between ethylene glycol and fourteen other compounds. It will be seen that the time required to swell halfway to equilibrium in molar solutions of ethylene glycol in sea water varied considerably, *i.e.* from 7.5 to 29.5 minutes, and averaged about twenty minutes as compared with an estimated time of possibly as many hours for glycerol and one to five minutes or less for most of the other compounds.

Glycerol (18 experiments) penetrates *Arbacia* eggs slowly, but at a measurable rate, as is shown in Fig. 1. The eggs cease to swell in hypotonic sea water in about an hour, but with glycerol present in the external solution they do not live long enough to attain their equilibrium volume; apparently more than twenty-four hours would be needed for the completion of the process. Several of the experiments summarized in Table I give actual figures for the times necessary to increase the volumes of the eggs by a definite amount in glycerol solutions, as compared with the corresponding values for water, formamide, acetamide, urea and ethylene glycol. It will be observed that of these substances glycerol is by far the most slowly penetrating, an hour being required in its solutions for a 5 per cent increase in cell volume, which occurs in less than half of that time in urea solutions and in less than three minutes in water or in solutions of formamide, acetamide or ethylene glycol.

Erythritol (3 experiments), the highest member of the series of polyhydric alcohols studied, does not enter *Arbacia* eggs to an appreciable extent either from pure solutions or in the presence of salts, during an exposure of five hours (Fig. 1).

4. SUBSTITUTION PRODUCTS OF GLYCEROL AND ETHYLENE GLYCOL

The replacement of one or more OH groups in glycerol by either a chlorine atom or by an acetic acid radical greatly increases the ease with which the compound enters *Arbacia* eggs, but at the same time increases its toxicity to the eggs.

Monacetin (9 experiments), in which one OH group of glycerol has been so replaced by an acetic acid radical, is the least toxic of the substitution products that were tested. The effect of the substitution in question is most striking, the time required for a 20 per cent increase in volume being reduced from an average value of 250 minutes to one of approximately two minutes. Monacetin enters eggs far more easily than ethylene glycol but somewhat more slowly (*i.e.*, one and one-half to two times) than diacetin (Fig. 3). The experimental results obtained varied so much that it was impossible to compare its behavior quantitatively with that of the monohydric alcohols. It appeared on the whole to penetrate more slowly than ethylene glycol mon- and diacetates, or than monochlorhydrin, all of which are rather toxic substances.

Diacetin (17 experiments). The addition of a second acetic acid radical still further increases the ease of penetration of the *Arbacia* egg. When made up in sea water, the rate of penetration of diacetin is very close to that found for ethyl alcohol, ethylene glycol mon- and diacetates and faster than that for monochlorhydrin and monacetin. It is also faster than that of any of the amides. The complete series: glycerol$<$monacetin$<$diacetin is represented in Fig. 3.

Monochlorhydrin (4 experiments). In this compound a chlorine atom replaces one hydroxyl group of glycerol, thereby increasing the penetrating power of the substance to an extent almost exactly the same as with monacetin. Monochlorhydrin is more toxic than monacetin, however, and this may perhaps account for part of the increase (Table III).

Ethylene Glycol Monacetate (4 experiments) and *Ethylene Glycol Diacetate* (2 experiments) were both tested, but satisfactory results could not be obtained because of their toxicity. It seems probable that they both penetrate rapidly, though not so readily as the figures in Tables III and IV would seem to indicate, since any toxic effect increases the apparent rate of penetration.

5. Sugars

Experiments with these compounds were difficult to perform in the usual manner since the eggs tended to float in the solutions. By using sufficiently dilute solutions of the sugars in sea water, however, it was possible to show that no measurable penetration occurred.

Sucrose (3 experiments), as far as could be judged from such measurements of diameter as are possible on floating eggs, failed to enter the cells from a pure molar solution. It also gave no evidence of penetration when made up to lesser concentrations in sea water.

Dextrose (7 experiments). Experiments with this substance were continued for eight hours or more without any trace of its penetration either in the presence or in the absence of salts in the external solution (Fig. 1).

6. Amides

The four amides used form an instructive homologous series; but, unfortunately, they prove to be rather difficult to work with. When molar solutions in distilled water are used, toxic effects invalidate the results. If the solvent is 60 per cent sea water, then it is necessary, in order to make the comparison a fair one, that all of the curves should end at the same equilibrium volume. Ordinarily this can be accomplished by simply repeating any experiment which does not come out at the proper level, but with the amides there are further difficulties of a chemical nature which may now be mentioned.

Formamide (33 experiments), in particular, gives solutions which are strongly acid in reaction. Adjustment of the pH necessitates the addition of so much NaOH that the concentration of non-penetrating substances in the external solution is definitely increased and the volume of the eggs at equilibrium correspondingly decreased. In one experiment in which the equilibrium value in a so-called molar solution of formamide in 60 per cent sea water was compared with those for a series of various dilutions of sea water, it was found that the final volume of eggs in the formamide solutions was the same as that in 68 per cent sea water instead of 60 per cent. On the other hand, if the solution is diluted so that its osmotic pressure is correct, then the concentration of formamide is decreased. Because of these difficulties, it is impossible to compare this substance satisfactorily with the other compounds. The figures given in Tables I, II and IV show the considerable variation that occurs. At times formamide seems to enter the eggs more rapidly than acetamide, but in other experiments more slowly.

Acetamide (17 experiments) gives solutions which are somewhat acid, but it does not present the extreme difficulties that were encountered with formamide. The penetration of acetamide was found to be more rapid than that of glycerol, urea and ethylene glycol, but slower than that of any of the other compounds studied, with the possible exception of formamide. The time required to reach the halfway point on the swelling curve is about ten minutes for molar solutions of this substance in 60 per cent sea water—a value only half of that for ethylene glycol (Tables I, II and IV), but nearly twice that ordinarily obtained with the least toxic of the more rapidly penetrating substances such as the monohydric alcohols.

Propionamide (11 experiments) enters *Arbacia* eggs more rapidly than acetamide (Tables I and II; Fig. 4) but at a slower rate than butyramide. It has not been compared directly with any compounds except the other amides and water, but the average values for the time in minutes for reaching a point halfway to equilibrium in such series was found to be: water, 4.5; butyramide, 4.9; propionamide, 6.3; acetamide, 10.3. Formamide is omitted from the series for the reasons already mentioned.

Butyramide (7 experiments) enters the eggs at a rate which is consistently faster than that for propionamide. Swelling in its solutions is, however, noticeably slower than in water alone. Results such as are given in Fig. 4 have been duplicated several times. Additional experiments are given in Table II.

7. Amino Acids

Glycocoll (4 experiments). Eggs left for twelve hours in solutions of glycocoll made up in 60 per cent sea water failed to show any indications of swelling.

8. Other Compounds

Urea (27 experiments) penetrates at a rate which is much the same as that of glycerol, though usually not quite so slow. The lower curves in Fig. 1 illustrate the swelling of the eggs in solutions of these two substances and Table I gives the actual times necessary for 5 per cent and 20 per cent increases in volume in solutions of each substance. It must be remembered in interpreting these figures that the slopes of the curves are very slight and small differences in slope will mean large differences in the times required to reach a given volume. Table I also gives comparative values for urea and water, formamide, acetamide and ethylene glycol. In Table III, additional comparisons are made, but urea experiments could not be continued long enough to obtain comparable figures.

The general behavior of urea is not that of an inert substance. Instead, it acts as if it had a definite effect, either on the membrane or on the protoplasmic consistency of the egg. Most of the eggs placed in pure urea solutions (molar in distilled water) lose their spherical shape after 20 to 50 minutes and become distinctly irregular. Later, " pseudopodia " may form and the egg may move much as an amoeba does. After a time it usually rounds up and becomes perfectly spherical again—only perhaps to put out " pseudopodia " once more, after a little while. This peculiar behavior of the eggs is considerably less marked if the urea solution is made up in sea water.

Ethyl urethane (3 experiments). This substance is so toxic to the eggs that no satisfactory results could be obtained with it. It probably penetrates rapidly. Reference to one experiment which was fairly good is made in Table I.

TABLE I

Time in minutes required for eggs to swell to a point halfway between their initial and equilibrium volumes. ( ) time required for a 5 per cent increase in size. [ ] time required for a 20 per cent increase in size. Substances made up to molar strength in 60 per cent sea water.

Date	Water	Methyl Alcohol	Ethyl Alcohol	Ethylene Glycol	Glycerol	Erythritol	Dextrose	Sucrose	Monacetin	Diacetin	Formamide	Acetamide	Propionamide	Glycocoll	Urea	Ethyl Urethane
7/12/29......	5.8			29.5												
7/18/29......	3.5			7.5												
7/19/29......	5.0			13.5												
7/20/29......	5.8			21.0 *												
7/22/29......	4.5			16.2												
7/23/29	3.8			14.0												
7/25/29......	4.1	4.1 *	4.1 *	17.0							5.2 †	4.5				
7/26/29......	3.7 4.8			15.5 17.0	∞						6.2 † 4.5 †					
7/31/29......	5.0 (1.8)				60 *						? (1.8)	8.3 (2.7)			(27) (17)	
7/10/30......	5.2			23.6												
7/13/30......	3.7			27.3												
7/16/30......	4.5			13.3												

* Value calculated by extrapolation of curve.

† Eggs not swelling to the same equilibrium volume as in other solutions.

TABLE I—(*Continued*)

Date	Water	Methyl Alcohol	Ethyl Alcohol	Ethylene Glycol	Glycerol	Erythritol	Dextrose	Sucrose	Monacetin	Diacetin	Formamide	Acetamide	Propionamide	Glycocoll	Urea	Ethyl Urethane
7/17/30......				(1.3)	(66)											
7/20/30......					slow	∞		∞								
7/21/30......				27	slow	∞	∞									
7/24/30......	2.6 3.2															5.5 3.5
7/25/30......				[4.8]	[143]										[100]	
7/26/30......					[309] [321]										[204]	
7/28/30......											8.0 †	11.7	5.8			
7/29/30......	3.6										10.5 †	9.2	5.7			
7/30/30......	3.0	4.1	3.2						5.8	2.7						
8/1/30.......											8.3	9.6				
8/9/30.......					[228]									∞		
8/10/30......												6.8	4.8	∞		

TABLE II

Time in minutes required for eggs to swell to a point halfway between their initial and equilibrium volumes. Substances made up to half molar strength in sea water.

Date	Water	Ethylene Glycol	Formamide	Acetamide	Propionamide	Butyramide
7/12/29. . .	5.8	23.5				
7/18/29. . .	3.5	6.2				
7/19/29. . .	5.0	14.0				
7/20/29. . .	5.8	14.2				
8/10/30. . .					6.6	4.0
8/11/30. . .	4.4		6.2 †		6.5	4.9
8/12/30. . .	4.7		9.7	10.5 11.0	5.7	5.7
8/13/30. . .	4.5		6.9	9.0 12.2	6.3	5.2 †
8/15/30. . .	2.8? 4.3		9.0? 9.7?	9.1	6.5	4.9
8/16/30. . .	3.7 3.6				6.1	

† Eggs not swelling to the same equilibrium volume as in other solutions.

TABLE III

Time in minutes required for eggs to swell to a point halfway between their initial and equilibrium volumes. ( ) time required for a 5 per cent increase in size. Substances made up to molar strength in sea water.

Date	Water	Methyl Alcohol	Ethyl Alcohol	Ethylene Glycol	Glycerol	Monacetin	Diacetin	Ethylene Glycol Monacetate	Ethylene Glycol Diacetate	Formamide	Acetamide	Mono-chlorhydrin	Urea
8/9/29. . . .		5.3	4.5	16.5	∞	3.1		1.7	1.0?	6.7 †	?	2.8	∞
8/10/29. . .	4.0	3.0	2.4	6.7	∞	2.4	1.7		1.7	4.1 †	?	2.7	(36)

TABLE IV

Time in minutes required to increase volume of eggs 50 per cent. Molar solutions.

Date	Methyl Alcohol	Ethyl Alcohol	Ethylene Glycol	Glycerol	Monacetin	Diacetin	Ethylene Glycol Monacetate	Formamide	Acetamide	Mono-chlorhydrin	Urea
7/8/29......					2.2	1.5					
8/5/29......	3.8	2.8		slow				8.8			slow
8/6/29......	4.0	3.0						8.6	6.3		slow
8/7/29......			11.5 *		4.3		3.2 *	6.7	5.2	2.3 *	
8/13/29......			7.0?					3.5	6.7		

The eggs were first shrunk in a similar solution of glycerol in all experiments except:

Table I	Table II	Table IV
7/12/29	7/12/29	7/8/29
7/10/30		8/5/29
7/13/30		8/6/29
7/20/30		8/7/29
7/21/30		
7/24/30		
8/ 9/30		

III

The results that have been presented are, on the whole, in good agreement with those of Overton (1895, 1907), Bärlund (1929), and others, on plant material. Certain substances, such as sucrose, dextrose, erythritol and glycocoll, fail to enter either plant cells or *Arbacia* eggs to an appreciable extent. Others penetrate at a measurable, but slow, rate. Glycerol and urea belong to this group. A third group of substances, including ethylene glycol, acetamide, and perhaps formamide and propionamide, penetrate both types of cells readily but at a rate somewhat slower than that which is characteristic of the remaining compounds. With the plant cells ethylene glycol enters even more rapidly than propionamide, but with *Arbacia* eggs it is the slowest of the four substances. A possible explanation of this difference will be discussed later.

The group of compounds which penetrate the *Arbacia* egg most rapidly includes butyramide, methyl and ethyl alcohols, monacetin and diacetin, and a number of other substances whose ready penetration may be due in part to toxic effects upon the cells. Of the substances in this group, monacetin appears to behave somewhat differently with plant cells, its rate of penetration into the cells of *Rhoeo*, according to Bärlund, being of the same order of magnitude as that of glycerol, though distinctly more rapid.

The results with *Arbacia* eggs are also in general agreement with those of Mond and Hoffmann (1928) for ox corpuscles, the chief difference in the two cases being in the very ready permeability of the erythrocytes to urea, which has also been noted by other workers, as compared with the slight permeability of the eggs.

As to the factors governing the permeability of the *Arbacia* egg, there have been found in this work no exceptions to Overton's principle that high lipoid solubility of a compound is associated with rapid penetration. As examples of the applicability of this principle the following series of closely related compounds, in which lipoid solubility and rate of penetration run parallel, may be mentioned:

glycerol < monacetin < diacetin.
acetamide < propionamide < butyramide.
methyl alcohol < ethyl alcohol.

It is probable, however, that factors other than lipoid solubility are also significant. Of such factors, the size of the molecule, especially of substances of low lipoid solubility, is thought by a number of workers to be of much importance. Bärlund (1929) and Mond and Hoffmann (1928) have even gone so far as to estimate the molecular size below which penetration is possible regardless of lipoid solubility. Expressed in terms of molecular refraction, which they accept as an approximate measure of molecular size, the critical values given are 15 for *Rhoeo* cells and 25 for the ox erythrocyte, respectively.

The substances of low lipoid solubility whose behavior with the *Arbacia* egg has been tested, arranged in the order of their molecular weights (the order of their calculated molecular refractions, according to Bärlund, is almost the same), are: sucrose, dextrose, erythritol, glycerol, glycocoll, ethylene glycol, urea, acetamide and formamide. Of these, the *Arbacia* egg is impermeable, or practically so, to all down to and including erythritol, slightly permeable to glycerol, impermeable to glycocoll, fairly permeable to ethylene glycol, much less so to urea and most permeable of all to acetamide and formamide.

While there is in this series a general parallelism between molecular weight (and molecular refraction) and penetrating power, two compounds in particular, glycerol and ethylene glycol, stand out of their proper positions. As a matter of fact, these compounds, though not freely lipoid-soluble, do possess such solubility in a higher degree than their immediate neighbors in the series and it is not improbable that their behavior may be due to this fact. The more rapid penetration frequently obtained with formamide, the substance of lowest molecular weight and molecular refraction in the list, as compared with that of the somewhat more lipoid-soluble acetamide, might be used as evidence of the importance of molecular size, were it not for the toxicity of the former substance and the difficulties mentioned above, connected with its use.

It has already been mentioned that ethylene glycol enters *Arbacia* eggs at a rate which is relatively slower than that at which it penetrates cells of *Rhoeo discolor*. This fact is of interest chiefly because the molecular refraction of ethylene glycol, *i.e.* 14.4, is very close to the critical refraction of 15 which Bärlund believes marks the upper limit in the size of molecules which can penetrate the plant cells by way of pores. Since ethylene glycol enters cells of *Rhoeo* more rapidly than does propionamide, the smaller size of its molecule may perhaps more than compensate for the greater lipoid solubility of the latter substance. But with *Arbacia* eggs the much slower penetration of ethylene glycol than of propionamide might perhaps suggest that its entrance by way of pores is distinctly limited and that if such pores are present in this type of cell their diameter averages somewhat less than that for the cells of *Rhoeo*. In general, it appears that for *Arbacia* eggs lipoid solubility is definitely an important factor in determining the rate at which any substance enters the cells. Molecular size also probably plays a part, though the evidence which has been obtained on this point is not considered to be conclusive.

IV

It was shown by Lillie (1916) that fertilized eggs of *Arbacia* swell more rapidly in hypotonic and shrink more rapidly in hypertonic sea water, than unfertilized eggs from the same female. These results were interpreted as indicating an increase in permeability to water following fertilization. Since no attempt appears to have been made to determine whether dissolved substances behave similarly, a number of experiments of the sort previously described were carried out with ethylene glycol.

In order that the results of such experiments may be strictly comparable, the various groups of cells must all approach the same average equilibrium volume. As mentioned above, it is not easy to select quickly, and without previous measurement, different groups which will do this exactly. It is necessary, therefore, to repeat the experiments until a suitable comparison can be made. As a consequence of this unavoidable difficulty only three sets of experiments, out of a much larger number actually performed, gave results which were considered to be entirely satisfactory. These experiments form the basis for Table VI and Fig. 5.

In Fig. 5 are represented the swelling curves obtained in one such experiment with fertilized and unfertilized eggs from the same individual, in 60 per cent sea water and in a molar solution of ethylene glycol in 60 per cent sea water. In each case there had been a preliminary shrinking of the eggs for ten minutes in a glycerol solution made up to the same concentration as the ethylene glycol, in order that no shrinkage should occur during the experiment proper. Within such a short period glycerol behaves practically as a non-penetrating substance.

It will be observed that in both solutions swelling is decidedly more rapid with fertilized than with unfertilized eggs. In the case of the hypotonic sea water, in accordance with Lillie's view, this may be interpreted as indicating a greater permeability of the fertilized eggs to water. But the fact that swelling in the ethylene glycol solution is faster with fertilized than with unfertilized eggs is not in itself a proof that the solute enters more rapidly in the former case, since the observed rate of swelling depends upon the rate of penetration of water as well as upon that of the solute.

A mathematical attempt to separate the effects of the respective rates of penetration of the solute and solvent in cases such as this has recently been made by M. H. Jacobs and will be published elsewhere. In the meantime, the data already obtained may be compared roughly by referring to Table V, in which are shown the times required for

Table V

A comparison of the times, in minutes, required by unfertilized and fertilized eggs to swell to a volume halfway between their initial and equilibrium volumes when placed in 60 per cent sea water and in ethylene glycol made up to molar concentration in 60 per cent sea water.

Date	Substance	Unfertilized Eggs	Fertilized Eggs	Unfertilized
				Fertilized
7/10/30.....	Water	5.2	2.7	1.9
	Ethylene glycol	23.6	11.0	2.1
7/13/30.....	Water	3.7	2.8	1.3
	Ethylene glycol	27.3	17.7	1.5
7/16/30.....	Water	4.5	3.4	1.3
	Ethylene glycol	13.3	9.0	1.5

fertilized and unfertilized eggs to reach a volume halfway between their initial and equilibrium volumes, where swelling involved (*a*) the entrance of water alone, and (*b*) the entrance of both water and solute. The ratios for the times in question for unfertilized and fertilized eggs are given in column 5. It will be observed that the ratio for the ethylene glycol solution is in each case greater than that for water alone. In other words, there is, with ethylene glycol present, a proportionately greater decrease in the time required for a given volume change after fertilization than is found in its absence. This evidence, as far as it goes, would seem to indicate an increased permeability to ethylene glycol itself, though the differences in the observed rates are not very large.

Attempts to carry out similar experiments with other substances were not entirely successful. It was fairly definitely determined, however, that the increase in permeability which presumably occurs upon fertilization was not sufficiently great to permit a measurable penetration into fertilized eggs of substances such as glycocoll, erythritol and dextrose to which the unfertilized egg appears to be almost or entirely impermeable.

It is a pleasure to acknowledge my indebtedness to Dr. M. H. Jacobs, at whose suggestion this work was begun and under whose direction it has been carried on; and to Dr. Balduin Lucké for his kindness in demonstrating his methods of handling and measuring the eggs.

SUMMARY

1. The permeability of the *Arbacia* egg has been tested by following the volume changes which occur in solutions of nineteen non-electrolytes.

2. The general behavior of this type of cell agrees fairly closely with that of the plant cells studied by Overton and by Bärlund and, to a somewhat lesser extent, with that of the mammalian erythrocyte.

3. No exception has been found to the principle that compounds which are freely lipoid-soluble readily penetrate the *Arbacia* egg. In the case of substances which are only slightly lipoid-soluble, the size of the molecule appears to be of importance, but unequivocal evidence on this point is difficult to obtain because of the existence of complicating factors of various sorts.

4. Some evidence has been obtained that following fertilization there is an increase in permeability to ethylene glycol.

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THE PERMEABILITY OF THE *ARBACIA* EGG TO AMMONIUM SALTS

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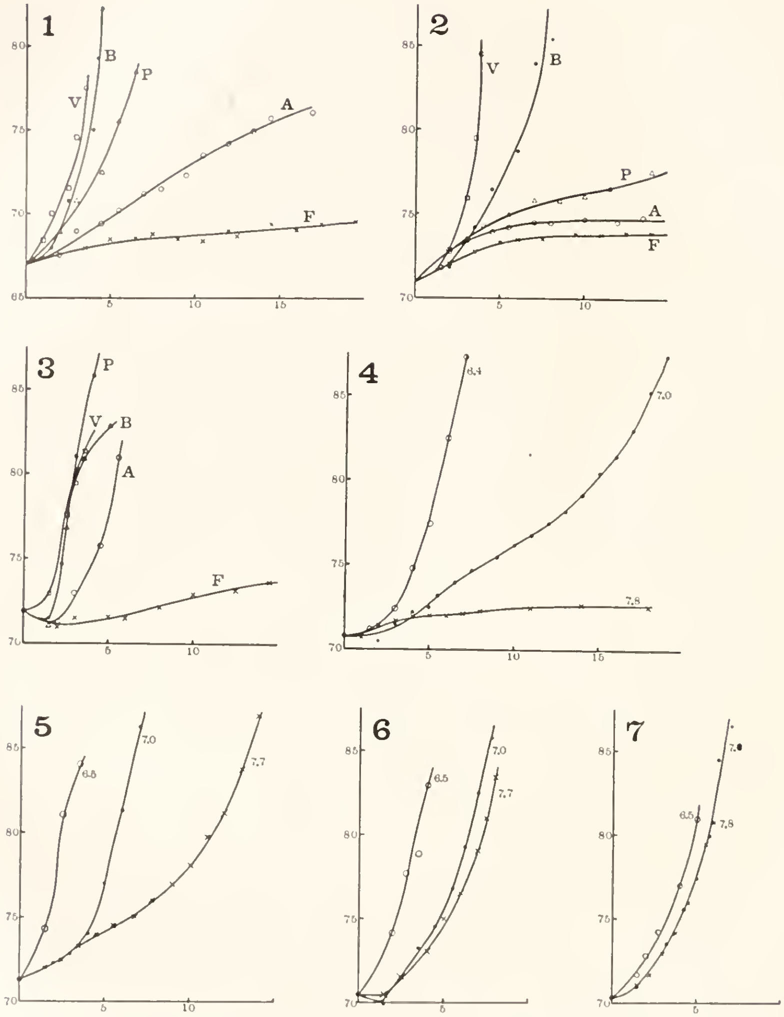
I

Salts, as a rule, appear to penetrate living cells with considerable difficulty. A striking exception to this behavior is found in the case of many ammonium salts which are known to enter mammalian erythrocytes with the greatest ease. (Gryns, 1896; Hedin, 1897). Jacobs (1927) has offered an explanation of this unusual property of the ammonium salts which is based on the behavior of the products of their hydrolysis in aqueous solutions. In the case of NH_4Cl, for example, the products of hydrolysis are largely undissociated ammonia, which enters all cells with the greatest ease, and highly dissociated hydrochloric acid, one of whose ions, Cl', is capable of being exchanged through the anion-permeable wall of the erythrocyte for OH'. The final result is a gradual accumulation within the cell of the salt in question, leading eventually to hemolysis. Other cells, which lack the pronounced specific permeability to anions that characterizes the erythrocyte, would not be expected to accumulate ammonium chloride, but only small quantities of free ammonia. In accordance with this view Jacobs finds that *Arbacia* eggs do not swell in isotonic solutions of ammonium chloride, the amount of ammonia which must penetrate in order to establish equilibrium with the external solution being too small to cause an appreciable volume change.

With the ammonium salts of the lower fatty acids, however, the situation is somewhat different. These salts form, upon hydrolysis, ammonia and free fatty acid. The latter is largely undissociated and is able to penetrate cells easily. Once inside them it unites with the ammonia, which has entered independently, forming salt again. Jacobs (1927) showed that the behavior of erythrocytes is in accordance with this theory, and he predicted that, since apparently all cells are permeable to both ammonia and the lower fatty acids, the ammonium salts of these acids should be able to penetrate any type of cell in this fashion. The present paper furnishes evidence that *Arbacia* eggs are freely



FIGS. 1–7: Swelling curves for *Arbacia* eggs in half molar solutions of various ammonium salts. Abscissæ, times in minutes; ordinates, diameters in arbitrary units each equal to 1.064μ. (See Footnote 1 concerning the pH values used.)

1. Ammonium formate (*F*), acetate (*A*), propionate (*P*), butyrate (*B*) and valerate (*V*); pH 7.0.
2. Same; pH 7.8.
3. Same; pH 6.5.
4. Ammonium acetate at pH indicated beside each curve.
5. Ammonium propionate at pH indicated beside each curve.
6. Ammonium butyrate at pH indicated beside each curve.
7. Ammonium valerate at pH indicated beside each curve.

permeable to these ammonium salts, and that the mechanism of the penetration is that predicted.

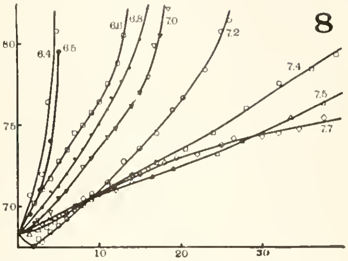


FIG. 8. The swelling of *Arbacia* eggs in M/2 solutions of ammonium acetate of the pH values indicated beside each curve. Abscissæ, times in minutes; ordinates, diameters in units each equal to 1.064μ. (See Footnote 1 concerning the pH values used.)

The solutions of ammonium salts were made up by adding to half normal NH_4OH sufficient concentrated acid to give the pH desired. The volume changes produced in this way were slight and the resulting solutions could be considered, with little error, to have a salt concentration of M/2, or approximately that of sea water. In some cases, where hypertonic solutions were desired, the salts were made up in bicarbonate-free sea water instead of distilled water. To obtain this, 2.4 cc. of normal HCl were added to a liter of sea water, and the CO_2 was driven off by aerating for twelve to fifteen hours. The pH was brought up to neutrality by the addition of a little NaOH and then the ammonium salt was prepared as before. The half molar solutions made up in distilled water were isosmotic with the cells, and penetration of salt from them could be detected by the swelling of the eggs, which proceeded until they were destroyed. The solutions in bicarbonate-free sea water, on the other hand, were initially hypertonic so that the eggs shrank, regaining their original size gradually only if the salt penetrated. The volume changes were studied by the method described in the preceding paper (Stewart, 1931).

II

The experiments were divided into two main series, one in which the ammonium salts were made up in pure M/2 solutions and another in which they were dissolved in sea water or half molar KCl. (NaCl could not be used because of its toxicity in unbalanced solutions.) The advantage of experiments of this second type lies largely in the fact that in them the eggs must approach, and then maintain, an equilibrium volume. Failure to do so gives definite evidence of injury to the cells.

This method was used as a check on the results obtained by the first one in which conditions were, on the whole, considerably more abnormal and for which no acceptable criterion of injury was available. The results obtained by the two methods were in good agreement.

For most of the experimental work the ammonium salts of the first five saturated fatty acids were used. Of the acids formed by the hydrolysis of these salts, four, namely acetic, propionic, butyric and valeric, are of very nearly the same strength. Furthermore, these four acids have dissociation constants that are almost the same as that of ammonia. Consequently at pH 7.0 there should be approximately equal amounts of free acid and ammonia present in the solutions. Formic acid is enough stronger so that the pH of the salt solution must be lowered to about 6.5 before equal quantities of acid and ammonia are obtained. For this reason the formate is not strictly comparable, at the same pH, with the other salts.

A comparison of the swelling curves of *Arbacia* eggs placed in M/2 solutions of the five salts at pH 7.0 (Fig. 1) shows the marked differences in the rates at which the salts enter the eggs. Since ammonia is known to penetrate these and other cells with extreme rapidity, it is reasonable to believe that the fatty acids, which in pure solutions enter cells somewhat more slowly, are to a certain extent the limiting factor which determines the rate of swelling. Because of the fact that at a given pH the concentration of free acid is approximately the same in the solutions of all of the salts except the formate, the swelling curves must give an indication of the relative order of penetration of the acids themselves. Numerous experiments indicate that the series is formic$<$acetic$<$propionic$<$butyric$<$valeric, which is the order of their lipoid solubilities, and the inverse of that for their molecular weights. Furthermore, it is the order frequently found in experiments with pure solutions of the acids, provided the results are not confused by injury effects. (For references to the literature see Jacobs, 1927).

The same order of penetration is observed if the salts are made up to half molar strength in either bicarbonate-free sea water or half molar KCl. Figure 9 gives a set of the curves obtained in experiments with sea water solutions. It will be noted that the valerate proves decidedly toxic, the eggs continuing to swell in it until they are cytolyzed. All of the other curves approach an equilibrium, however, indicating the lack of any decided injury to the cells. The fact that the equilibrium volume is not exactly the same as the initial volume of the eggs may be due to slight osmotic differences in the solutions themselves or may perhaps indicate the existence of secondary factors of some sort whose effects are of relatively minor importance.

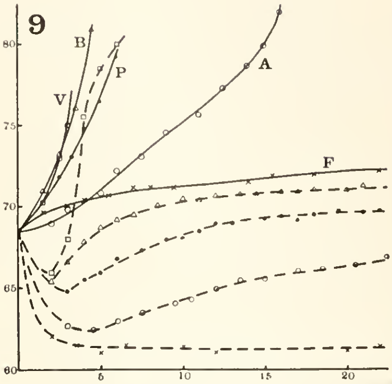


FIG. 9. Swelling curves for *Arbacia* eggs in solutions of ammonium formate (×), acetate (△), propionate (◎), butyrate (●) and valerate (□); pH 7.0. Solid lines, M/2 solutions; broken lines, M/2 solutions in bicarbonate-free sea water. (See Footnote 1 concerning pH values used.)

III

If the rate of entrance of free acid into the cell is the most important factor in influencing the rate of penetration of the salt, then it would be expected that the pH of the solution, which determines the relative concentrations of free acid and ammonia, would have a very important influence upon the swelling process. This proves to be the case. In general the increase in volume of the cells tends to become more rapid as the pH is lowered, down to about 6.0, and to become progressively slower as the pH is increased above 7.0. The swelling of the eggs in a solution of ammonium acetate at pH 7.8 is almost imperceptible (Fig. 2), while at pH 6.5 (Fig. 3)—an acidity which is not in itself markedly injurious—they increase in size so rapidly that cytolysis occurs within five or six minutes. A striking illustration of this effect of pH in changing the amount of free acid, and consequently the rate of swelling of the *Arbacia* eggs, is given in experiments such as that represented in Fig. 8. Here ammonium acetate solutions were used and the pH varied, in small steps, between 7.7 and 6.4. There is no exception to the orderly increase in rate with decrease in pH. The experiment has been repeated several times, always with the same results. Similar, though less extensive, observations have been made with each of the other salts (Figs. 5, 6, 7). The only essential difference between them lies in the spread of the three curves, which becomes less and less as the ease of penetration of the free acid involved increases, until finally, with the valerate, the curves are not separated from each other by an amount that is significantly greater than the error of the method. As a result the three valerate curves do not maintain any definite relation to each other. Frequently penetration at pH 7.8 appears to be faster than at 7.0, or the curve for pH 6.5 is a trifle less steep than that for 7.0. But in about half of the experiments the order was found to be the same as that for the other salts, namely pH 6.5 > pH 7.0 > pH 7.8.

The effect of variations in pH is thus seen to parallel the changes in the amount of undissociated acid that is present in the solution. As has been mentioned previously, at pH 7.0 approximately equal quantities of free acid and ammonia are present. At a more alkaline reaction the amount of acid would be less than that of ammonia, and vice versa. Since the acid, rather than the ammonia, tends to be the limiting factor in determining the rate of swelling of the eggs, it is relatively easy to diminish this rate by a change of the pH in the alkaline direction. It is conceivable that at a sufficiently alkaline reaction almost no swelling would occur. This proves to be the case with an ammonium formate solution at pH 7.8, in which eggs often failed to swell appreciably during a 30-minute exposure. This failure was not due to any injury to the eggs, for after much longer exposures than that they were capable of giving typical swelling curves in an acetate solution. Attempts were made to obtain similar results with the other salts by raising the pH beyond 7.8, but toxic effects became so marked that the experiments could not be carried out.

As the pH is lowered below 7.0, more and more acid is present in the solution and swelling becomes increasingly rapid. If the pH is lowered sufficiently, the amount of ammonia might theoretically become so small that it would in turn assume the rôle of the limiting factor. This actually seems to be the case with the ammonium acetate. The maximum rate of swelling for this salt was obtained at about pH 6.0, while below that point the rate decreased until at pH 5.5 the curve obtained was almost identical with that at pH 6.5 or 6.6. Similar experiments with the other salts were impracticable because of the high toxicity of their more acid solutions.

As would be expected from the marked effect of pH upon the amount of free acid present in the solution, the divergence between the rates of swelling for the five salts is most marked in the alkaline range. Here the acids themselves are the chief factor in determining the rate, and differences in their ability to enter the eggs become most prominent. At pH 7.8 (Fig. 2) the penetration of the formate is sometimes almost impossible to detect, while the valerate enters so rapidly that cytolysis occurs in three minutes. At lower pH values, however, where the acids are present in comparative abundance and where the ammonia becomes a more important factor, the difference would be expected to be less. Reference to Fig. 3 shows that as a matter of fact the spread of the curves at pH 6.5 is much reduced, the three for the propionate, butyrate and valerate being almost superimposed.

IV

In addition to the experiments which have been performed with the ammonium salts of fatty acids, a considerable number have been carried out with other ammonium salts and with some sodium and potassium salts of fatty acids. With ammonium nitrate and ammonium chloride no change occurred, within half an hour, in the volume of eggs placed in M/2 solutions, though eggs from the same lot were found to swell appreciably in ammonium formate at pH 7.7 during that time. Indeed, eggs may be left in ammonium chloride made up in bicarbonate-free sea water for 80 minutes without any change in size and at the end of this time give normal swelling curves if transferred to a neutral acetate solution. In other words, they appear not to have been injured appreciably by the chloride and their failure to swell is probably due to the fact that the acid cannot enter the cell and so no salt accumulates.

An attempt was made to work with solutions of ammonium benzoate and salicylate, but they proved very toxic to the eggs and it was not possible to obtain reproducible results with them. About all that can be said concerning these salts is that they both seem to enter the cells rapidly, though this apparent speed of penetration may be entirely due to the effects of injury which are ordinarily apparent within ten minutes. Ammonium lactate, on the other hand, enters very slowly, there being no appreciable penetration by the end of an hour from M/2 solutions at pH 7.7 or 7.0, though at pH 6.5 the volumes have increased to an extent that is just barely significant. At pH 7.0 a 10 per cent increase in volume occurs in about three hours as compared with a corresponding time of approximately five minutes for a similar solution of ammonium propionate. Since lactic acid differs from propionic acid only in the substitution of an OH group for a hydrogen atom, this behavior illustrates the marked effect upon permeability of the introduction of a polar group into a molecule.

Repeated experiments with sodium or potassium acetate and with sodium valerate at varying pH levels failed to show any penetration of these substances—or at least any penetration sufficient to cause a change in cell volume.

I wish to thank Dr. M. H. Jacobs, under whose direction this study has been made, for his many suggestions and his never-failing interest in the progress of the work.

SUMMARY

1. The rate of swelling of *Arbacia* eggs in solutions isosmotic with sea water of the ammonium salts of the first five saturated fatty acids has been found to be in the order: valerate > butyrate > propionate > acetate > formate.

2. In solutions of these salts made up in bicarbonate-free sea water or in M/2 KCl recovery of the original volume after a preliminary shrinkage follows the same order.

3. Within the pH range from approximately 7.8 to 6.2 the rate of swelling increases with increasing acidity. Under certain conditions a further lowering of the pH may reverse the effect.

4. No significant changes in volume occur in solutions of ammonium nitrate or chloride, though cells exposed to such solutions for over an hour are still capable of swelling in ammonium acetate.

5. These results agree with the theory suggested by Jacobs, that there is no appreciable penetration of either the salt or its ions as such but that undissociated ammonia and fatty acid, formed by hydrolysis of the salt, penetrate the cell separately, uniting again after their entrance.

6. The rate of entrance of the acid, rather than that of the ammonia, usually appears to be the limiting factor in determining the rate of swelling of the cell.

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[1] Since the salt errors of the indicators for the solutions used are not exactly known, it has been thought best to use the uncorrected pH values throughout this paper, particularly since relative rather than absolute figures are of importance. The proper corrections may be made at any time when the necessary data become available. Since these corrections would probably be about — 0.2, the values here given are too high by approximately 0.2 pH units. The standards used for comparison were the ordinary M/15 phosphate buffer solutions.

NOTES ON TREMATODES FROM A LONG ISLAND DUCK WITH DESCRIPTION OF A NEW SPECIES

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In view of the fragmentary knowledge concerning the trematodes of North American birds, and the extent to which these parasites are distinct from those of other continents, it seems proper to record certain observations which we have made.

Examination of the alimentary tract of a single duck, sold in the New York market, yielded four species of trematodes, belonging to four different genera. One of the species is new to science and two others are recorded from America for the first time. The high degree of infestation is unusual, since previous examinations have shown that ducks bought in the market are rarely parasitized by trematodes. Furthermore, the conditions under which most Long Island ducks are raised and prepared for market tend to prevent the acquisition of such parasites. These facts indicate that the bird was either a wild duck or that it had not been closely confined in breeding and feeding pens.

To substantiate the correctness of specific determination, brief descriptions and figures of the parasites are included.

Echinostoma revolutum (Froelich, 1802) Looss, 1899

Three specimens from the intestine are referred to this species. The one shown in Fig. 1 measures 13 mm. in length and 1.9 mm. in width. Measurements of internal structures are as follows: oral sucker, 0.245 mm.; pharynx, 0.18 mm.; acetabulum, 0.635 mm.; ovary, 0.43 mm.; testes, 0.795 mm. long by 0.43 mm. wide. The collar of spines is not complete in any of the specimens and consequently their number can not be determined with certainty. In other features, however, the worms agree so well with the descriptions of *E. revolutum* as given by Looss (1899), Lühe (1909), and Dietz (1910) that there appears to be no doubt concerning their identity. *E. revolutum* has been previously reported from North America and the life cycles of the species was experimentally demonstrated by Johnson (1920).

Psilochasmus oxyurus (Creplin, 1825) Lühe, 1910

Since the description of Creplin (1825) this species has been studied by von Linstow (1882), Braun (1902), Lühe (1909), and Odhner

PLATE I

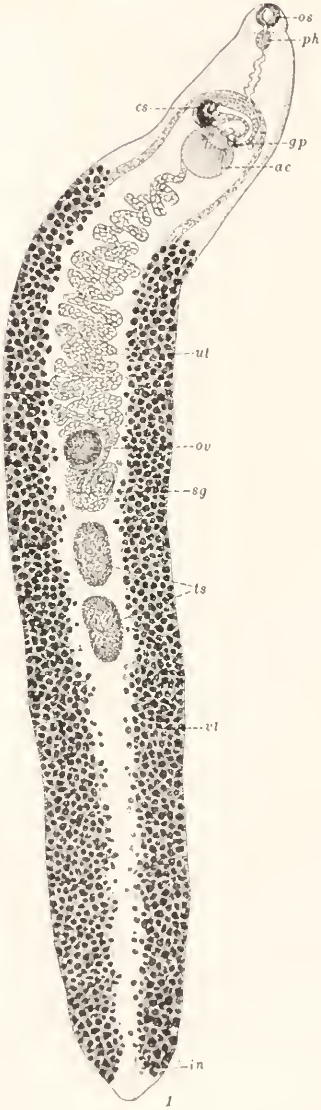
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FIG. 1. *Echinostoma revolutum*

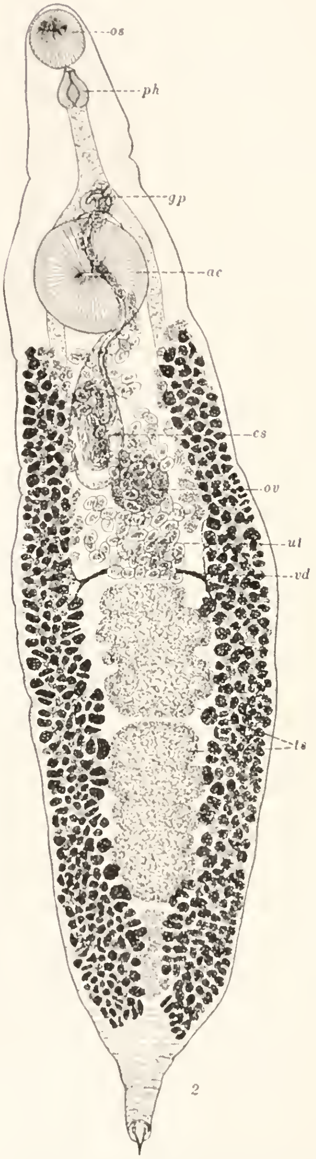
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FIG. 2. *Psilochasmus oxyurus*

Abbreviations

ac	acetabulum	*ov*	ovary	*ts*	testis
cs	cirrus sac	*ph*	pharynx	*ut*	uterus
gp	genital pore	*sg*	shell gland	*vd*	vitelline duct
in	intestine	*sv*	seminal vesicle	*vt*	vitellaria
os	oral sucker				

(1913). The material used in these investigations came from various species of ducks taken in Northern Europe. A second species, *Psilochasmus longicirratus,* was described by Skrjabin (1913) from *Fuligula nyroka* in Russian Turkestan. A comparison of his description with those of other authors, especially that of Odhner, and with the specimens at hand shows no specific differences. The only feature in which there is not actual overlapping is the length of the cirrus sac and reports do not agree on this point. Braun stated that the cirrus sac is long and slender with a seminal vesicle in its base. Odhner found the cirrus sac ending a short distance before the ovary, while in the specimen shown in Fig. 2 the sac extends to the ovarian level. Consequently, there is no means of distinguishing between the species, and *P. longicirratus* may be regarded as a synonym of *P. oxyurus.*

Four specimens from the duck, one of which is shown in Fig. 2, show essential agreement with the description of *P. oxyurus* and accordingly are assigned to that species. The worms measure from 4.6 to 5.5 mm. in length and from 1 to 1.25 mm. in width. The shape of the body is characteristic and the terminal spike projects from a depression in the ventral surface at the caudal end. The spike is composed of strong muscular fibers, continuous with those of the body wall. The acetabulum is situated at the anterior fourth or fifth of the body and measures from 0.54 to 0.6 mm. in diameter.

The oral sucker is 0.3 to 0.32 mm. in diameter and followed almost immediately by a muscular pharynx about one-half its size. The esophagus is lined with epithelium and bifurcates at the level of the genital pore to form the intestinal ceca. The ceca terminate blindly just anterior to the caudal lobes of the vitellaria.

The testes are distinctly lobed. They measure from 0.653 to 0.884 mm. in length and 0.461 to 0.530 mm. in width. They are situated one behind the other in the anterior part of the caudal half of the body. A vas deferens arises from the anterior, ventral surface of each and passes forward, the duct from the anterior testis on the left and that from the posterior testis on the right side of the body. The left duct passes under the ovary and both open into the caudal end of the cirrus sac. This portion of the sac is expanded and filled with spermatozoa. From the seminal vesicle the ejaculatory duct makes a coil and then passes in a straight course to the genital pore.

The ovary measures from 0.27 to 0.32 mm. in diameter. It is slightly left of the median plane, about two-fifths of the distance from the cephalic testis to the acetabulum. The oviduct arises at the caudal end and passes backward to the öotype, which is enclosed in a large "shell gland." From the öotype Laurer's canal leads to the dorsal surface. The vitellaria are lateral, dorsal and ventral, and extend from the level of the acetabulum almost to the posterior end of the body. The lobes of the two sides are connected by a ventral commissure behind the testes, and just in front of the cephalic testis ducts arise from the ventral sides and pass dorsally and medially, meeting on the dorsal side to form a common vitelline duct which passes forward to open into the öotype. There is no seminal receptacle, but the initial portion of the uterus is filled with masses of spermatozoa. The uterus passes in a sinous course, backward to the level of the cephalic testis and then forward to the genital pore, situated below the bifurcation of the alimentary tract. Eggs in the uterus measure from 0.1 to 0.12 mm. in length and from 0.07 to 0.077 mm. in width.

Notocotylus gibbus (Mehlis in Creplin, 1846) Kossack, 1911

This species, unknown from its discovery by Mehlis until the original specimens were restudied by Kossack (1911), has not been reported since that time so far as we can determine. The European specimens were found in the intestine of *Fulica atra* and *Gallinula chloropus*. Other species are known from Europe and two species, *N. urbanensis* and *N. quinqueserialis* have been described from North America. The specimens from the Long Island duck agree most closely with the description of *N. gibbus* and apparently belong to that species.

Fifteen specimens (Fig. 3) were recovered from the washings of the alimentary tract but their original location in it is uncertain. Since members of the Notocotylidæ ordinarily infest the ceca and terminal part of the intestine, it is probable that these worms also were in that region. They are much flattened and sexually mature specimens measure from 1.1 to 1.5 mm. in length by 0.5 to 0.6 mm. in width. The ventral surface bears three rows of pits, 12–14 in each. Those near the ends of the body are smaller than those near the middle.

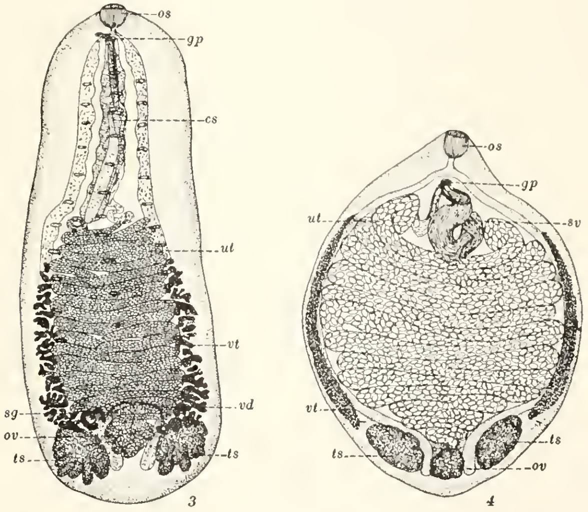
The oral sucker is usually broader than long and measures from 0.07 to 0.11 mm. in diameter. The esophagus is short and bent, opening almost immediately into the ceca. These structures end blindly between the testes.

The testes are deeply lobed, 0.2 to 0.23 mm. in length and 0.12 to 0.14 mm. in width. Ducts from the testes unite in front of the öotype to form a common duct which passes forward on the dorsal side of the body. It becomes coiled as it approaches the middle of the body and increases in size to form a seminal vesicle. Immediately in front of the uterine loops it enters the cirrus sac, which measures from 0.43 to 0.5 mm. in length.

The ovary lies between and in front of the testes. It is deeply lobed and measures from 0.13 to 0.15 mm. in diameter. The öotype complex lies anterior to the ovary and is of the usual notocotylid type. Eggs measure from 0.02 to 0.024 mm. in length and 0.01 to 0.012 mm. in width. They bear long filaments at both poles.

The points of difference between the description of Kossack and the present specimens appear to be only variations that might be expected in a single species. His statement that the testes are some distance from the posterior end of the body and that the intestinal ceca are relatively close together may be explained by difference in sexual maturity. In specimens containing fewer eggs than the one shown in Fig. 3, the testes are relatively farther forward and the ceca closer together. In fact, in sexually immature, non-gravid worms, the testes are proportionally farther forward than in the specimen shown in Kossack's figure. It appears that when the uterus becomes filled with eggs the other structures are somewhat displaced. Kossack reported that the ventral glands are very weakly developed and that they number 6–8 in each row. Since their location is not shown in his figure, one can not tell whether they were present in a section of the body or distributed uniformly throughout its length. The material available to Kossack was probably in poor condition and the observations were ap-

PLATE II



Abbreviations as in Figs. 1 and 2

FIG. 3. *Notocotylus gibbus*
FIG. 4. *Paramonostomum parvum*

parently not checked by study of serial sections. It seems very probable, therefore, that some of the ventral glands were overlooked.

Paramonostomum parvum n. sp.

The genus *Paramonostomum* was erected by Lühe (1909) to contain a single species, *Monostomum alveatum* (Mehlis in Creplin, 1846), that occurs in several European birds. Earlier accounts of the species are reviewed in the paper by Kossack (1911).

Barker (1916) questioned the validity of the genus on the ground that it differs from *Notocotylus* only in the absence of ventral glands. Careful examination shows, however, that the absence of ventral glands is only one of a number of fundamental differences which distinguish the genus. In shape of body, location and proportion of internal organs, extent of uterus, and form of the copulatory organs, there are marked differences between *Notocotylus* and *Paramonostomum*.

Harrah (1922) described a second species, *Paramonostomum echinus,* from the intestine of the American muskrat, *Fiber zibethicus.* Review of his description and figure shows that, in regard to the features listed above, his specimens do not agree with *P. alveatum,* type of the genus. In these respects they are similar to *Notocotylus.* It appears, therefore, that *P. echinum* should be removed from the genus *Paramonostomum.* It may be regarded as a type of a new genus or the limits of *Notocotylus* may be extended to contain it. The second of these alternatives would probably invalidate the genus *Catatropis.*

Twenty specimens from the intestine of the Long Island duck agree very closely, except for size, with *P. alveatum.* They may prove to be only a variety of that species but the difference is too great, in the absence of intermediate sizes, to assign them to it, and they are regarded as a new species for which the name *Paramonostomum parvum* is proposed.

Sexually mature specimens are broadly ovate, almost as broad as long, concave ventrally, pointed anteriorly, and rounded posteriorly. They measure from 0.25 to 0.5 mm. in length and from 0.2 to 0.35 mm. in width. There are no papillae on the ventral surface, and the presence of spines on the cuticula is doubtful.

The oral sucker measures from 0.035 to 0.046 mm. in diameter. There is no pharynx, the esophagus is short, and the ceca pass backward in a wide arc, close to the lateral edges of the body. Near the posterior end of the body the ceca turn medially and then pass backward between the testes and the ovary, terminating about the level of the caudal margin of the ovary.

The testes are oval, lobed organs, situated in the extracecal areas at the posterior end of the body. They measure from 0.08 to 0.095 mm. in length and from 0.06 to 0.07 mm. in width. The vasa deferentia unite in front of the oötype to form a common sperm duct that passes anteriad. At the caudal end of the anterior third of the body the sperm duct opens into a large, much coiled seminal vesicle. The cirrus sac is curved, 0.035 to 0.06 mm. in length and 0.025 to 0.04 mm. in width. The caudal part of the cirrus sac contains a small section of the seminal vesicle.

The ovary is situated in the median plane, near the posterior end of the body. It is a much lobed structure, 0.05 to 0.07 mm. in diameter. The " shell gland " is anterior to the ovary and about one-half its size. The female genital complex is similar to that of *Notocotylus*. The uterus extends in transverse folds to the caudal end of the anterior third of the body and other coils extend farther forward on either side. The metraterm is short and opens at the genital pore, situated in the median plane immediately behind the bifurcation of the alimentary tract. The vitelline glands consist of small follicles which occupy the extracecal areas from the level of the testes to that of the seminal vesicle. Eggs measure from 0.021 to 0.024 mm. in length and from 0.011 to 0.013 mm. in width. They are provided with long polar filaments.

Discussion

The discovery of these trematodes in a North American duck affords supplementary data concerning two little known species, previously found only in Europe, and adds a second species to the genus *Paramonostomum,* which supports the validity of that genus. It supplements other data which tend to indicate that the parasitic fauna of Europe are being introduced into North America and that exotic species are being established here. Migratory animals, especially birds, may acquire infections in one location and during their journeys distribute infective stages of the parasites over wide areas. The presence of suitable intermediate hosts, and of conditions which permit their infestation, result in the spread of parasites to new locations. In many instances the parasites are able to utilize new intermediate and definitive hosts and thus extend both their geographical range and list of host species. It appears that there is more overlapping of the parasitic faunas of the Eastern and Western hemispheres than has been realized.

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THE PRODUCTION OF NORMAL EMBRYOS BY ARTIFICIAL PARTHENOGENESIS IN THE ECHIUROID, *URECHIS*

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INTRODUCTION

The eggs of annelids and mollusks have been the subject of numerous parthenogenesis experiments, but only a few instances have been reported in which normal development was said to have been obtained. Lefevre (1907) described and figured parthenogenetically produced embryos of *Thalassema mellita* which resembled the normal, but he states (p. 116), "I have never observed a single instance where the trochophores rose to the surface of the water." Just (1915) reports the production of "normal-looking swimming forms—trochophores scarcely to be distinguished from the normal"—from eggs of *Nereis* exposed to high temperature. Morris (1917) obtained embryos which she described as "fairly normal swimming larvæ" from eggs of *Cumingia* exposed to high temperature and hypertonic sea water. In other cases the embryos obtained were described merely as swimmers or even uncleaved swimmers, (Allyn, 1912; Loeb, 1908, 1912; Lillie, 1910; Kostanecki, 1908; Bullot, 1904; Scott, 1906; Treadwell, 1902; Fischer, 1903). It is evident from the above citations that normal embryos have rarely, if at all, been obtained. The question may then be raised as to whether the sperm performs some special function in the eggs of annelids and mollusks, such that in its absence normal development is less likely to be obtained.

In the eggs of some annelids and mollusks there is evidence that the entrance point of the sperm is instrumental in determining the plane of bilateral symmetry of the embryo (Just, 1912; Morgan and Tyler, 1930). Such an orienting point is lacking, of course, in eggs treated with the usual parthenogenetic agents. It is important to know, therefore, whether eggs, in which the entrance point of the sperm is a factor in the determination of bilateral symmetry, are capable of normal development after artificial activation.

For the eggs of *Urechis* it was found that the first cleavage plane coincided with the entrance point of the sperm in the great majority of cases. Since in spirally cleaving eggs the first cleavage plane bears a definite relation to the plane of bilateral symmetry of the embryo, it may be safe to conclude that the sperm entrance point determines the orientation of the embryo in *Urechis*. The eggs of *Urechis* can be activated by means of certain artificial agents; such as, hypotonic sea water, hypertonic sea water and high temperature. Top-swimming embryos indistinguishable from those produced by normally fertilized eggs were obtained from eggs treated with hypotonic sea water. However, the normal embryos were produced in very small numbers, the various types of abnormal swimmers being far more abundant.

The eggs which are to cleave and develop can be distinguished and isolated from the others at a few minutes after treatment. The manner in which these eggs respond to the treatment presents a striking contradiction to the view (Just, 1922) that normal development results only when the initial response of the egg to the artificial agent is similar to its response to the sperm.

Material and Methods

Urechis caupo was found at Monterey Bay and described by Fisher and MacGinitie (1928). Professor MacGinitie later found the same species at Newport Bay and I am indebted to him for the information on the location of the animals and the method of obtaining the sexual products from them. This remarkable echiuroid is ripe practically the year round, and one pair of animals may supply eggs and sperm for a number of experiments. The ability to obtain eggs repeatedly from the same individual would seem to cut down the variability of different experiments. However, it was found that the eggs of a segmental tube, from which samples had previously been removed, decrease in size after a couple of weeks and their response to fertilization is not quite 100 per cent, as is the case when eggs are removed from an untouched tube.

The agents used to activate the eggs were diluted sea water, distilled water, concentrated sea water and high temperature, but since the first was mostly used in these experiments, it alone will be described. The dilutions of sea water were made up of distilled water and sea water taken at a definite height of tide. The eggs were washed twice and allowed to settle in a centrifuge tube. The supernatant sea water was then removed and the eggs together with 0.3 to 0.5 cc. of sea water transferred to 50 cc. of the diluted sea water. Eggs were then removed after various intervals of time to 10 cc. of sea water by means of a small bore pipette filled to 0.2 cc. Any dilution of sea water up to 80 per cent was found to be capable of activating the eggs. Controls of unfertilized eggs kept in covered dishes gave no activation and controls of eggs inseminated at the time of the experiment gave 95 to 100 per cent fertilization and development.[1]

NORMAL FERTILIZATION AND DEVELOPMENT

The development of the fertilized egg of *Urechis* is so similar to that of *Thalassema* (Torrey, 1902, 1903) that only a brief description need be given here for comparison with the parthenogenetic eggs. The "fertilization reaction" differs somewhat from that described for other marine ova, and since use was made of this reaction in the entrance point observations, it will be described below.

The unfertilized egg of *Urechis* (Fig. 1) has a rather unique shape. It has a large indentation which varies somewhat in size in different eggs. This shape is similar to that which a spherical rubber ball would assume if it were evacuated and filled with a volume of water about one-fourth less than $\frac{4}{3}\pi R^3$. The ratio of surface to volume is thus much greater than for a spherical egg. Eggs with two indentations (Fig. 2) may also be found, but if such eggs are made to round out in dilute sea water and returned to normal sea water only one indentation reappears.

The unfertilized egg contains a large germinal vesicle and a single nucleolus. The germinal vesicle is nearest the surface of the egg at the innermost point of the indentation. This point, as will be shown below, marks the pole of the egg, and the egg may be seen to be radially symmetrical about this axis (Fig. 3).

The sperm enters the egg at any point on the surface with respect to the indentation. Within three minutes after the attachment of the sperm, a clear conical process appears on the egg immediately below the point of attachment.[2] The cone enlarges within the next two minutes and the membrane immediately above it becomes thin (Fig. 4). At this time the indentation begins to round out and the fertilization membrane begins to separate from the surface of the egg. The sperm head then enters the cone (Fig. 5) and in about 90 seconds passes into the egg (Fig. 6), the tail remaining behind. During the next five minutes the cone continues to enlarge and becomes cylindrical rather than cone-shaped, with a rounded outer end (Fig. 7). It enlarges more rapidly than the perivitelline space, apparently stretching the membrane above it. The "cone" then begins to narrow considerably in width so that at about thirteen minutes after the entrance of the sperm it has the appearance of a single filament

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[1] The insemination was usually done in another part of the room by Betty S. Tyler.

[2] This time schedule holds for 20° C., unless otherwise stated.

(Fig. 8). It finally disappears at about seventeen to twenty-two minutes after the penetration of the sperm. The egg thus supplies a marker of the entrance point which can be seen as late as twenty minutes after insemination. Entrance point observations are therefore quite easy to make.

The first polar body is extruded 34 minutes after fertilization and the second at 44 minutes. Cleavage begins at 74 minutes after fertilization.[3] The first two divisions are equal. At the third cleavage the first quartet of micromeres arises dexiotropically. They are very slightly smaller than the macromeres. In the formation of the embryo, no essential differences were found from Torrey's description for *Thalassema*.

The Indentation and the Pole of the Egg

It is generally conceded that the polarity of an egg is established in the ovary, the polar axis being indicated by the eccentric position of the nucleus and radial arrangement of cytoplasmic materials about it. In the egg of *Urechis* the germinal vesicle is nearest the surface at the innermost point of the indentation (Fig. 1). If the indentation

Plate 1

Photomicrographs of the living objects. The magnification is 330 diameters for all the figures with the exception of Fig. 1, for which the magnification is 300 diameters.

Fig. 1. Unfertilized egg in "side" view, showing indentation, large germinal vesicle and nucleolus.

Fig. 2. Unfertilized egg in "side" view showing two indentations.

Fig. 3. Unfertilized egg, antipolar view.

Fig. 4. Formation of fertilization "cone" at point of attachment of sperm, and rounding out of indentation.

Fig. 5. Showing sperm within cone, beginning of dissolution of germinal vesicle and elevation of membrane.

Fig. 6. Showing sperm head half-way in egg, enlargement of "cone," and further elevation of membrane.

Fig. 7. Showing persistence and enlargement of the "cone" five minutes after entrance of sperm head into egg.

Fig. 8. Showing the shrinkage of the "cone" into a filament-like process, thirteen minutes after entrance of sperm, and disappearance of germinal vesicle except for nucleolus.

Fig. 9. Showing eccentric position of germinal vesicle in an egg in which the indentation was made to disappear by allowing it to swell in 50 per cent sea water.

Fig. 10. Showing migration of remains of germinal vesicle to pole as marked by indentation; entrance point of sperm shown by filament-like cone near antipole of egg fifteen minutes after entrance of sperm.

Fig. 11. Photographed in the same position as in Fig. 10; shows formation of first polar body at center of formerly indented area 32 minutes after fertilization.

Fig. 12. Photographed in the same position as in Fig. 11; shows formation of second polar body next to first; 44 minutes after fertilization.

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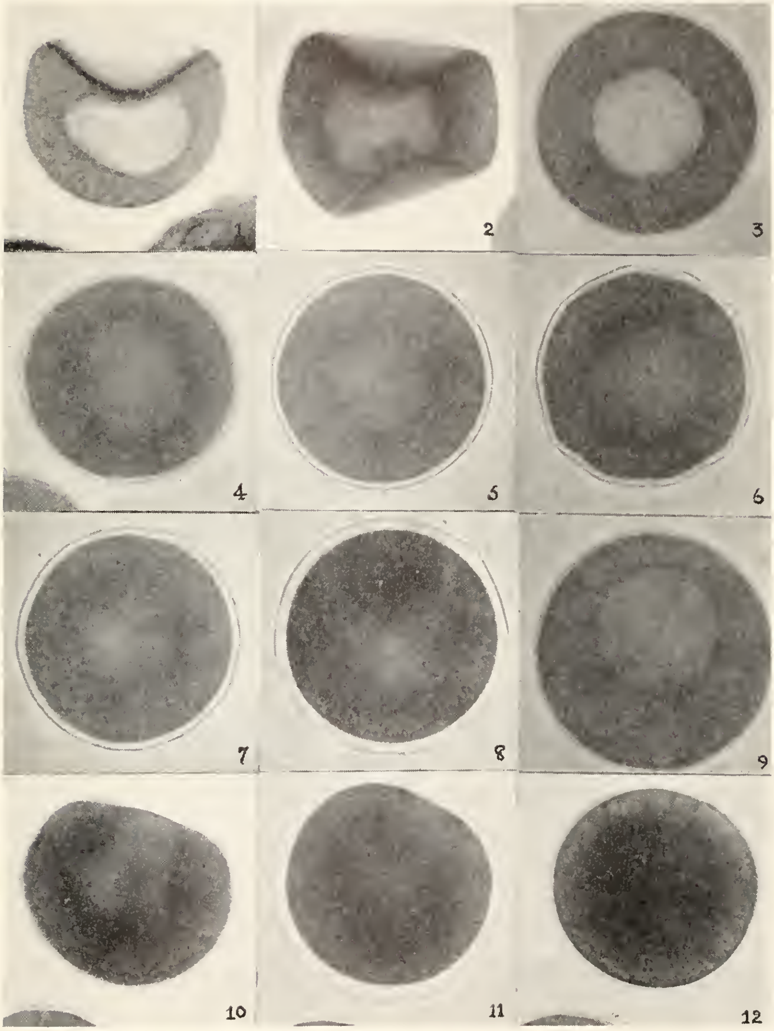
[3] At room temperature, which is generally about 23.5° C., the time for first cleavage is 65 minutes.

PLATE I.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is caused to round out by placing the egg in 50 per cent sea water, the germinal vesicle is seen to occupy an eccentric position in the egg (Fig. 9). The rounding out can be followed under the microscope, and the results of such observations show invariably that the eccentricity is the same as that of the original indented egg. This would indicate that the indentation marks the pole of the egg.

In order to obtain a more definite check on this relation, the point of extrusion of the polar bodies was determined on eggs in which the location of the indentation had been previously noted. The method used was identical with that described below for the entrance-point observations. The eggs selected for observations were those in which the indentation was on the side and thus distinctly visible. They were followed almost continuously throughout the time of rounding up and membrane elevation. Errors due to rotation of the egg were also partly guarded against by the presence of extra sperm which served as markers on the surface of the membrane. As the egg rounds up, the remains of the germinal vesicle are seen to migrate towards the surface of the egg in the region of the former indentation (Fig. 10). The first polar body later appears at about the center of that area on the surface (Fig. 11), and the second polar body is later extruded next to it (Fig. 12). The results of the observations given in Table I show four exceptions. These presumably represent cases in which the egg rotated within the membrane. However, there does not seem to be any general tendency for such rotation, since the polar bodies may appear in any position on the surface of the egg and remain in that position.

TABLE I

Relation between Indentation and Point of Extrusion of Polar Bodies

Divergence	No. of Eggs
0°– 10°	24
10°– 90°	2
90°–180°	2

RELATION OF THE ENTRANCE POINT OF THE SPERM TO THE PLANE OF BILATERAL SYMMETRY

The method used for making entrance point observations and the precautions to be taken have been described by Morgan and Tyler (1930). As has been mentioned above, the point of entrance of the sperm is very easily located in *Urechis* even as late as twenty minutes after fertilization. The first polar body appears ten minutes after the disappearance of the "cone" and serves as a marker on the surface of the egg. This diminishes the chances of unobserved rotation of the egg occurring.

The eggs were placed within a square of vaseline on a slide and the drop sealed over completely with a coverslip to prevent evaporation. This transfer was generally done at about ten minutes after fertilization. The first drawings were made five to ten minutes later, at which time the fertilization membrane has lifted off to about its full extent and the egg itself has become spherical. The position of the polar bodies was then noted as they appeared. At the time of cleavage the egg was watched almost continuously, since there is a tendency for eggs that are elongating vertically to roll over.

A number of the observations were made by Mr. Jackson Gregory, Jr., one of the students working at the Kerckhoff Marine Laboratory, and are listed separately in Table II as Series 1. The results of the observations given in this table show a coincidence of 71 per cent between the entrance point of the sperm and the first cleavage plane.

TABLE II

Relation between Entrance Point and First Cleavage Plane

Divergence	No. of Eggs		
	Series 1	Series 2	Total
0°–10°	61	66	127
10°–45°	13	24	37
45°–90°	8	6	14

This value is obviously very much higher than would be expected on a pure chance basis and clearly indicates that the position of the first cleavage plane is determined by the point of entrance of the sperm. The exceptions may be due to the inability of completely removing all sources of error or to the presence of abnormal eggs. The most convincing cases are those in which the entrance point and the polar axis lie in a horizontal plane on the slide. In order for coincidence to be obtained in such cases the egg must elongate vertically and the cleavage plane must cut through horizontally. Twenty-six eggs of that type were followed and in every case the first cleavage plane passed through the entrance point, although seven of them rotated during the division.

In eggs with spiral cleavage the plane of bilateral symmetry is given by the position of the 4*d* cell, which also marks the dorsal side of the embryo. The position of this cell is determined by the plane of first cleavage. In *Urechis* it can be located after its bilateral division into M_1 and M_2, and its position is such as to cause the plane of bilateral symmetry to make an angle of about 60 degrees with the first cleavage plane.

Parthenogenesis Experiments

Activation by Dilute Sea Water

The eggs of *Urechis* may be activated by dilutions of sea water ranging from 80 per cent to distilled water. Table III gives the time range for the different solutions over which activation may be obtained. It is taken from a set of experiments run at temperatures between 20 and 22.5° C. The third column gives the time of exposure at which activation begins, taken arbitrarily as 5 per cent activation. These values are obtained graphically by connecting the two nearest points above and below 5 per cent with a straight line. The fourth column gives the time of exposure necessary to obtain 100 per cent activation. With longer exposures the percentage of activation drops again to zero for certain concentrations of dilute sea water. Column five gives the exposure at which the activation drops to 5 per cent, calculated in the same manner as for column three. Solutions below 45 per cent sea water cause cytolysis of the egg, the time for cytolysis varying inversely with the amount of dilution.

Table III

Range of Exposure Time in which Activation May be Obtained

Dilution of Sea Water	Temperature	Calculated Time for 5% Activation	Time for 100% Act.	Calculated Time for 5% Activation
per cent	° C.	*seconds*	*seconds*	*minutes*
0	21.8	1	12	
20	22.0	3	20	
30	22.1	10	30	
40	22.0	11	50	
45	20.1	21	60	12
50	22.5	15	60	10
55	20.1	43	100	10
60	21.5	35	105	15
65	22.3	48	150	16
70	21.2	64	180	19
75	20.8	87	210	9
80	22.5	74	210	

It is readily seen from Table III that the time for activation decreases with increased dilution of the sea water. This is consonant with an interpretation of activation based on the volume of water taken in, since water enters more rapidly the greater the dilution of sea water in which the egg is placed.

Two other points may be mentioned here which, together with their interpretation, will be reported in detail in a subsequent paper. The increase in activation occurs much more rapidly than the decrease, giving a skew activation-time curve. The percentage of the activated eggs which cleave varies with the time of exposure; but its variation is such that as the percentage of activation increases the percentage of cleavage decreases. Thus, when 100 per cent activation is obtained no cleavage occurs, while to either side of that exposure time the percentage of cleavage increases.

PRODUCTION OF NORMAL EMBRYOS

Many of the cleaved eggs develop into swimming embryos, and a few into top swimmers, some of which are indistinguishable from those produced by normally developing fertilized eggs. Some of the experiments in which normal embryos were obtained are listed in Table IV.

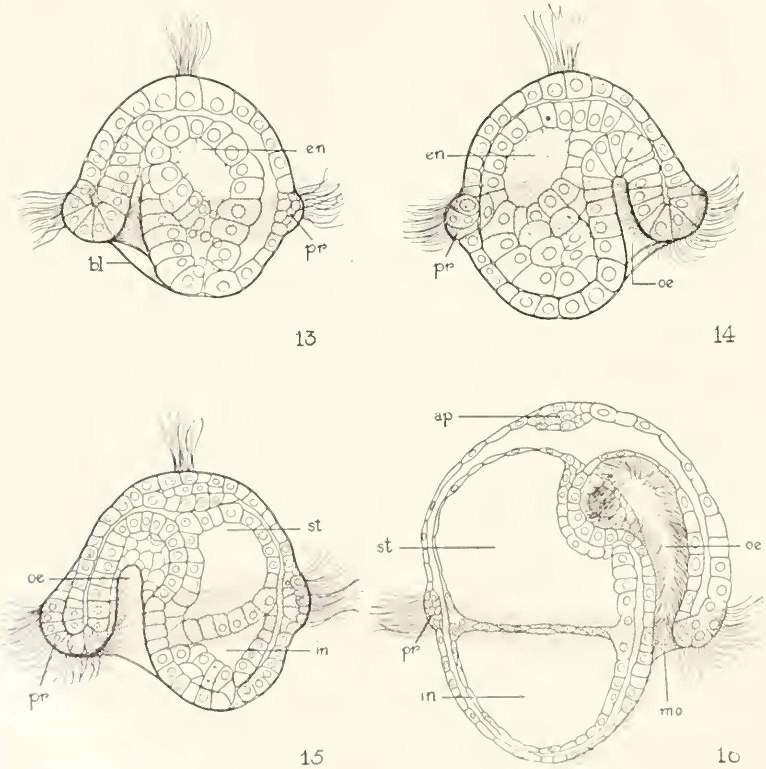
TABLE IV

Dilution of Sea Water	Exposure		Top Swimming Embryos	
			Normal	Abnormal
per cent	*minutes*	*seconds*		
30	0	20	3	25
	1	10	1	14
	1	0	3	29
	1	40	2	15
	2	20	5	41
40	0	20	2	11
	0	40	1	28
	1	0	1	14
	2	0	2	25
45	0	30	3	32
	1	15	1	15
50	1	30	2	8
	3	0	3	12
	6	0	3	17
60	4	0	3	26
	8	0	5	5
65	1	45	2	12
	4	0	1	5

As is shown by the table, the normal embryos are not produced by any particular dilution of sea water or any definite time of exposure. The percentage of normal development obtained is very small, most of the normal embryos listed occurring in dishes of five hundred to a thousand cleaved eggs.

In Figs. 13–16 some normal parthenogenetically produced embryos of various ages are presented. The embryos of fertilized eggs are not figured, inasmuch as they are identical with the parthenogenetic ones. The early gastrulation stages of the parthenogenetic embryos were not obtained, since at that time the normal embryos are not readily distinguishable from certain abnormal types to be described later.

The young trochophore of sixteen hours (Fig. 13) shows a large



Figs. 13–16.

ap, apical plate; *bl*, anterior portion of original blastopore; *en*, enteron; *in*, intestine; *mo*, mouth; *oe*, œsophagus; *pr*, prototroch; *st*, stomach. Normal embryos from artificially activated eggs; drawn from total mounts of preserved specimens.

Fig. 13. Sixteen-hour trochophore.
Fig. 14. Twenty-hour trochophore.
Fig. 15. Twenty-four-hour trochophore.
Fig. 16. Forty-eight-hour trochophore.

enteric mass in which a small cavity has appeared. The prototroch is well developed and the beginning of the œsophageal invagination is present below the prototroch on the ventral side of the embryo. In the twenty-hour trochophore (Fig. 14) the invagination has progressed towards the anterior end of the embryo and the post-trochal region has enlarged. The enteric cavity soon becomes divided into stomach and intestine (Fig. 15), and the œsophagus reaches the anterior end of the embryo. The whole embryo enlarges considerably during the next 24 hours and becomes a relatively thin-walled structure (Fig. 16). The anterior end of the œsophagus is turned to the right side of the stomach where it acquires a ciliated opening into the latter.

Bilateral symmetry is evident in the sixteen-hour embryo. The plane of bilateral symmetry is, as pointed out above, determined very much earlier, and for fertilized eggs, at the time of entrance of the sperm. The parthenogenetically activated eggs can evidently establish a plane of bilateral symmetry without the aid of a localized external agent such as the sperm entrance point. However, this is done in relatively very few instances, while in the great majority of cases, even with apparently normal cleavage, abnormal embryos of various types are obtained. Of particular interest in this connection is the occurrence of embryos that are more or less radially symmetrical.

Radially Symmetrical Embryos

Among the top swimming and bottom swimming embryos produced from the artificially activated eggs, there occur a number of forms which may be grouped in a radially symmetrical class. They retain their radial form until their death, which occurs long after the attainment of bilateral symmetry in the normal embryos.

Certain of these embryos have the form of a hollow blastula (Fig. 17), resembling the blastula of the sea-urchin. The prototroch is well developed and large entoblast cells are present below it, but no gastrulation occurs at any time in its life history. Another type (Fig. 18) shows the beginning of gastrulation, which in some instances progresses to the formation of a large enteric mass (Fig. 19). In certain cases the enteric cavity may be formed (Fig. 20). In none of these types is there any outward evidence of bilateral symmetry.[4] The blastopore does not undergo its antero-ventral migration, and the œsophagus is not formed.

It is quite probable, although the cell-lineage has not been worked out, that these forms arise from eggs in which the normal bilateral cleavages do not take place. Torrey (1903) has described eggs of *Thalassema* that have more or less completely reverted to the radial type. He states, "There is, also, strong reason for believing that this type never develops into an adult; for I found a few gastrula stages in which no *X* group, no *M* cells, no larval mesenchyme cells from the third quartet and no large anterior œsophagoblast $(2b_{2.2+})$ could be distinguished." It may be fairly safe to assume then that the cell lineage of the radial embryos described above is similar to that described by Torrey, and that the failure to develop into a bilateral trochophore results from the failure of certain bilateral cleavages to occur, so that no *X* group, *M* cells, etc. are formed.

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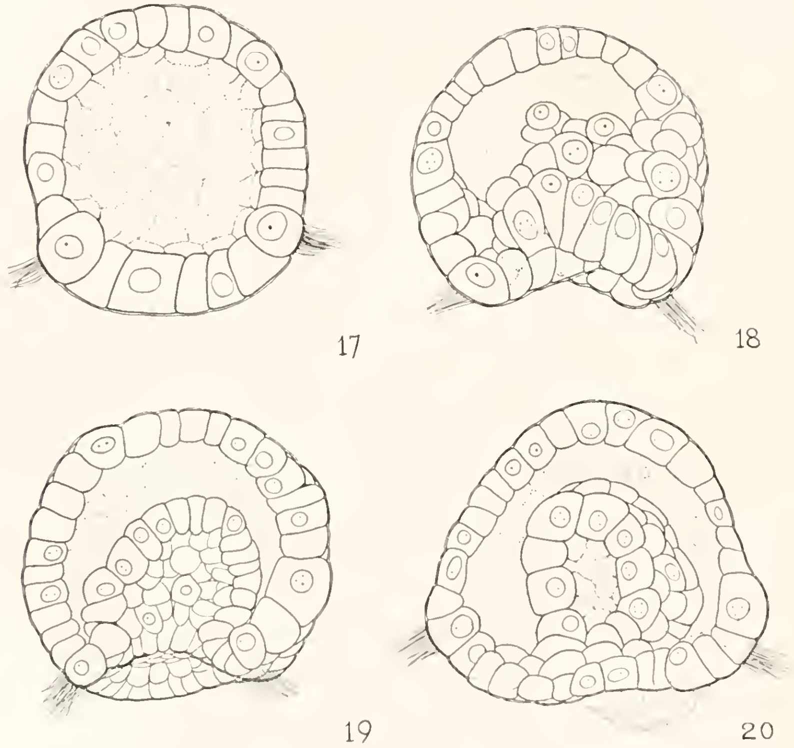
[4] Other forms have been observed which might be classed as transition types, but their abnormal form makes them quite difficult to analyze.



FIGS. 17–20. Radially symmetrical embryos from artificially activated eggs; drawn from total mounts of preserved specimens.

FIG. 17. Hollow blastula of 24 hours.

FIG. 18. Beginning gastrula of 22 hours.

FIG. 19. Gastrula of 28 hours, showing large enteric mass.

FIG. 20. Gastrula of 28 hours, showing enteric cavity.

Approximately one-quarter of the top swimming embryos and about 5 per cent of the bottom swimmers were radially symmetrical or approached the radial type. No radial embryos were observed among the trochophores of the normally fertilized eggs. Their occurrence as a result of artificial activation may be taken as an expression of the inability of the parthenogenetically activated egg to completely negotiate its problem of bilateral symmetry. The low percentage of normal development obtained is then accountable by the assumption that it is a matter of chance whether the response of the egg is similar to that resulting from the entrance of the sperm or whether it responds in an "abnormal" fashion to the artificial treatment. Before this could be put in more concrete terms, we would have to know much more about the response of the egg to the sperm in cases where the entrance point determines the median plane of the embryo.

Maturation and Cleavage of the Artificially Activated Eggs

It has been previously noted that 100 per cent activation may be obtained with certain times of exposure to different dilutions of sea water, but that the percentage of cleavage decreased with increased activation so that at the optimum point practically no cleavage is obtained. The eggs at the optimum point were all found to extrude two polar bodies,[5] whereas on either side of that exposure time increasing numbers of eggs with one and with no polar bodies were found. Eggs were therefore isolated according to the number of polar bodies formed in order to determine which type cleaved and developed.

Table V

Relation between Cleavage and Number of Polar Bodies Formed

Type of Egg Isolated	Number of Eggs Cleaved	Number of Eggs Uncleaved
No polar bodies	876	156
One polar body	0	83
Two polar bodies	0	518

The results obtained by isolating the various types of eggs are given in Table V. The eggs were isolated at 45 to 75 minutes after treatment and cleavage recorded about three hours later. A binocular microscope magnifying 125 diameters was used in making the isolations and the eggs were rolled around to make sure that the presence of polar bodies was not overlooked. None of the eggs with one or with two polar bodies were found to divide, whereas 85 per cent of the eggs with no polar bodies divided. This result is in accord with that obtained by Morris (1917) on the eggs of *Cumingia.* She found that a high percentage of maturation resulted in a low percentage of cleavage stages and larvæ, and from 1215 eggs with no polar bodies obtained five larvæ, whereas from 313 eggs with one or two polar bodies one embryo developed that was evidently uncleaved.

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[5] The first polar body may divide in about half of the eggs, as is the case in the fertilized eggs, so that when eggs with two polar bodies are referred to in the text it is meant to include also those with three polar bodies.

In *Urechis* the eggs with one or with two polar bodies can be readily distinguished from those with none. The former are decidedly more "normal" in appearance and in their reaction to the treatment than are the latter. It is quite surprising then that only the abnormal-looking eggs with no polar bodies develop, whereas the others do not even cleave. These types of eggs will now be described in some detail.

When the eggs are placed in the dilute sea water, the indentation rounds out and the whole egg swells due to the intake of water (Fig. 9).[6] Upon return to normal sea water the egg shrinks and the indentation reappears. The egg thus returns to its original shape. This is true for all the eggs so treated regardless of their subsequent history.

PLATE II

Photomicrographs of living objects. The magnification is 330 diameters.

FIG. 21. Artificially activated egg five minutes after return to normal sea water, showing beginning of membrane elevation and rounding of indentation.

FIG. 22. Artificially activated egg ten minutes after return to normal sea water, showing beginning of dissolution of germinal vesicle.

FIG. 23. Artificially activated egg fifteen minutes after return to normal sea water. Compare with Fig. 10 of fertilized egg.

FIG. 24. Artificially activated egg 34 minutes after return to normal sea water, showing first polar body.

FIG. 25. Artificially activated egg 47 minutes after return to normal sea water, showing second polar body.

FIG. 26. Artificially activated egg 50 minutes after return to normal sea water, showing single giant polar body.

FIG. 27. Artificially activated egg 45 minutes after return to normal sea water, showing presence of indentation, disappearance of germinal vesicle, and absence of membrane and of polar bodies.

FIG. 28. Antipolar view of an egg of the type shown in Fig. 27, showing small nucleus.

FIG. 29. Same egg as in Fig. 27, 40 minutes later, showing rounding up of egg, elevation of membrane, and formation of cleavage spindle (indicated by clear area) at right angles to polar axis.

FIG. 30. Same egg as in Fig. 27, 5 minutes later, completely rounded up.

FIGS. 39 AND 40. Show reappearance of indentation in its original position when egg is returned to normal sea water after treatment with dilute sea water.

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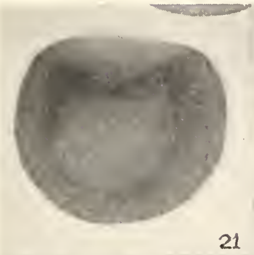
[6] In dilutions of sea water above 65 per cent the indentation does not completely round out.

PLATE II.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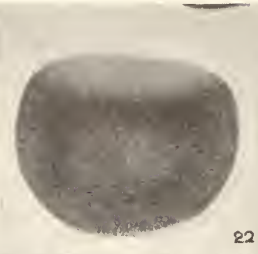
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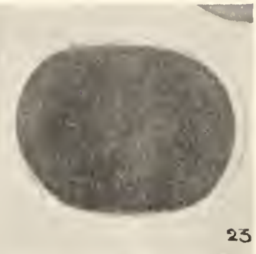
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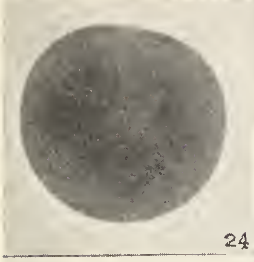
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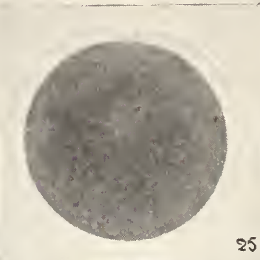
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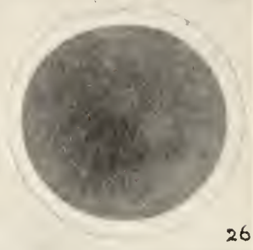
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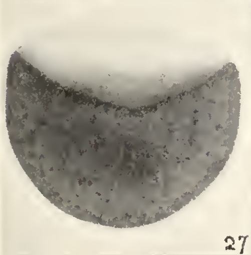
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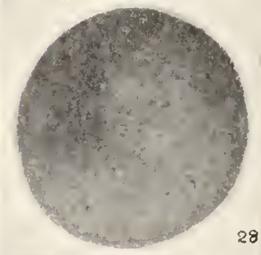
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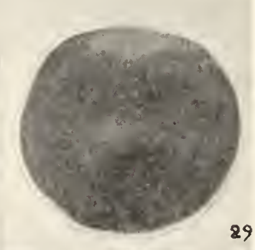
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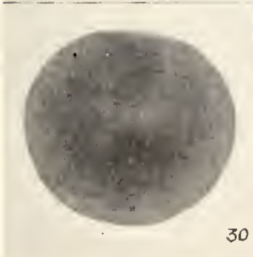
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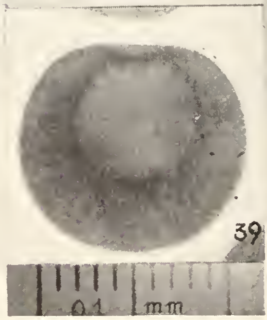
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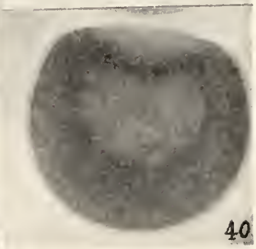
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It has been noted above that with certain lengths of exposures to dilute sea water, the time of treatment depending upon the concentration employed, 100 per cent of the eggs become activated. Such eggs upon return to normal sea water behave very much as though they had been normally fertilized except that no entrance cone is formed. The germinal vesicle breaks down, the indentation rounds out, and the membrane is elevated very much in the same manner as in the fertilized eggs (Figs. 21, 22, and 23). The first and second polar bodies (Figs. 24, 25) appear at the same time as they do in the fertilized eggs, if allowance is made for the time of return to normal shape in sea water.

This last point is illustrated in Table VI. The figures given in columns four and six of the table are for the time of appearance of the polar bodies from the beginning of the treatment. If allowance is made for the time of exposure, the time for appearance of the polar bodies still increases with the length of exposure. We must therefore subtract an amount equal to the time for return to original shape. A rough determination of the time of shrinkage shows it to be about

TABLE VI

Time of Polar Body Extrusion in the Artificially Activated Eggs

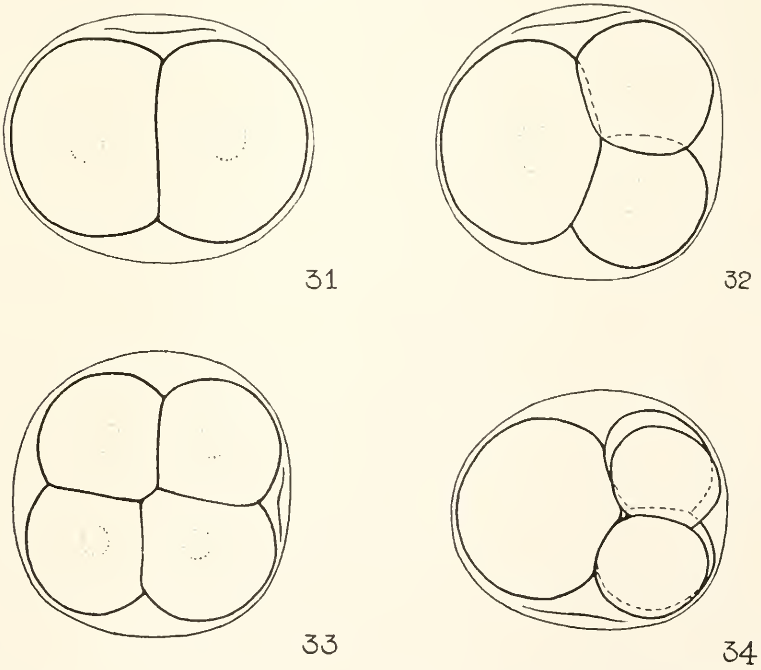
Dilution of Sea Water	Temperature	Length of Exposure	Time for First Polar Body		Time for Second Polar Body	
			Total	Calculated	Total	Calculated
per cent	° C.	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
30	20.0	⅓	35	34½	46	45½
		1	35	33½	47	45½
		2½	36	32¼	50	46¼
		4½	41	34¼	55	48¼
Fert. eggs				34		44
45	23.5	⅓	30½	30	41	40½
		1	31	29½	42	40½
		2½	33	29¼	43	39¾
		4½	36½	29¾	48	41¼
Fert. eggs				30		40
50	19.5	1	36	34½	48	46½
		1⅔	37½	35	49½	47
		2½	38	34¼	51	47¼
		4½	39¼	32½	52½	45¾
Fert. eggs				34½		46

* The total time is taken from the beginning of the treatment.

† The calculated time is that obtained by allowing for the swelling and shrinking of the egg.

half the swelling time. Allowing for that, the time of appearance of the polar bodies is comparable with the time of polar body extrusion in the fertilized eggs. This holds for all eggs that form two polar bodies, whether or not they have received the exposure resulting in 100 per cent activation. It is evident from these results that development does not start in the dilute sea water but after its return to normal sea water. This is in accord with the fact that the egg first assumes its original form on return to normal sea water and then proceeds to develop. It is also consistent with the fact (to be reported in a subsequent paper) that eggs which have been "over exposed" to dilute sea water do not become activated although they are still capable of fertilization.

The eggs which extrude only one polar body occur in much fewer numbers. The maximum amount observed was about one per cent of the activated eggs. When only one polar body is extruded it is



Figs. 31–34. Early cleavages of artificially activated eggs, drawn from photographs of living objects.

Fig. 31. Two-cell stage of an artificially activated egg.

Fig. 32. Three-cell stage of an artificially activated egg.

Fig. 33. Four-cell stage of an artificially activated egg, showing polar cross-furrow.

Fig. 34. Five-cell stage produced by the egg shown in Fig. 32.

generally quite large, being approximately equal in size to both polar bodies of the normal egg (Fig. 26). The average time of extrusion of the polar body was 45 minutes, which is the time of second maturation division in the normal egg.

The eggs that produce no polar bodies respond to the treatment in such a way that they were first classified as "poorly activated." The germinal vesicle disappears much more slowly than in the others. The rounding out of the indentation and elevation of the membrane may not occur for one to two hours. Figure 27 shows such an egg 45 minutes after treatment. Although the germinal vesicle has disappeared, no membrane is present and the indentation remains.

A small nucleus is later formed which persists until the time of rounding up of the egg (Fig. 28). The time at which the egg rounds up varies from 40 minutes to over two hours. As the indentation disappears an irregular membrane is lifted off from the surface of the egg (Figs. 29 and 30) and a spindle develops at right angles to the egg axis. Within 20 minutes after the rounding up, the egg elongates and divides (Fig. 31).

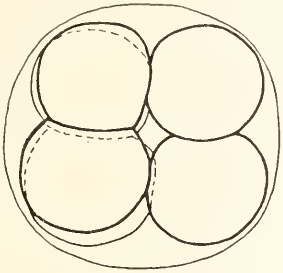
The first division may be equal or unequal. Of the 876 divided eggs listed in Table V, 485 had formed an equal or nearly equal two-cell stage. Four of the eggs had divided at once into three cells and the rest had formed an unequal two-cell stage. The next division of both the equally cleaved and unequally cleaved eggs may be into three cells (Fig. 32) or into four cells (Fig. 33). The time of division is quite irregular. Table VII gives the time schedule of division for 300 eggs without polar bodies that were isolated before cleavage began. The table shows that the behaviour of the eggs with respect to time of division is far from uniform; the second division in some eggs may occur even before the first division in others.

TABLE VII

Number of Eggs in Different Cleavage Stages at Various Times after Treatment—from Isolation of Uncleaved Eggs

Time after Treatment		1-cell	2-cell	3-cell	4-cell
hours	*minutes*				
1	10	300	0	0	0
1	20	298	2	0	0
1	35	270	30	0	0
1	45	267	31	2	0
2	0	202	96	2	0
2	30	112	160	24	4
3	0	30	201	41	28

The eggs which divide into three cells at the second division generally divide into five at the next (Fig. 34), the original undivided cell still remaining undivided. Those which divide into four cells at the second division may divide into six, seven, or eight cells at the third cleavage (Figs. 35, 36, 37, 38). The third cleavage is dexiotropic as in the normal egg. The four-cell stage also shows evidence of spiral cleavage in the presence of the cross furrow, which results from the *A* and *C* blastomeres being higher than the *B* and *D*. The time of third cleavage is also quite variable. In order to determine whether a more uniform result might be obtained, equally cleaved eggs were isolated according to whether they divided early or late. The results of two such experiments are given in Tables VIII and IX. No significant differences in types of cleavage obtained are evident, and no greater uniformity in time of division results, when the divided



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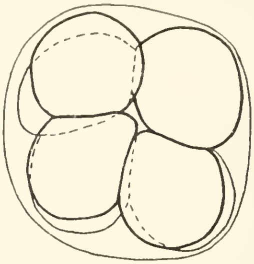

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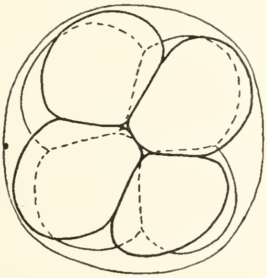

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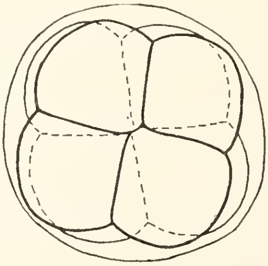

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Figs. 35–38. Early cleavages of artificially activated eggs, drawn from photographs of living objects.

Fig. 35. Six-cell stage produced by the type of egg shown in Fig. 33.

Fig. 36. Seven-cell stage produced by the type of egg shown in Fig. 33.

Fig. 37. Eight-cell stage produced by the type of egg shown in Fig. 33; showing dexiotropic cleavage.

Fig. 38. Later eight-cell stage showing dexiotropic cleavage.

TABLE VIII

Number of Eggs in Different Cleavage Stages at Various Times after Treatment—from Equally Cleaved Eggs Isolated Early

Time after Treatment	2-cell	3-cell	4-cell	5-cell	6-cell	7-cell	8-cell
1 hour 50 min. to 2 hrs. 15 min.......	190	0	0	0	0	0	0
2 hours 30 min......	168	22	0	0	0	0	0
2 " 47 "	143	47	0	0	0	0	0
2 " 53 "	129	47	14	0	0	0	0
3 " 0 "	106	60	24	0	0	0	0
3 " 5 "	106	50	24	10	0	0	0
3 " 10 "	92	59	26	10	0	3	0
3 " 30 "	56	48	39	22	6	11	8
4 " 0 "	21	36	31	38	22	17	25

eggs are isolated early or late. The normal-looking eggs in the eight-cell stage were isolated from the early dividing and late dividing series. The embryos produced were examined about 32 hours later.

TABLE IX

Number of Eggs in Different Cleavage Stages at Various Times after Treatment—from Equally Cleaved Eggs Isolated Late

Time after Treatment	2-cell	3-cell	4-cell	5-cell	6-cell	7-cell	8-cell
2 hours 20 min. to 2 hrs. 45 min.......	207	0	0	0	0	0	0
2 hours 52 min......	184	0	23	0	0	0	0
3 " 0 "	159	25	23	0	0	0	0
3 " 15 "	72	86	49	0	0	0	0
3 " 35 "	64	86	57	0	0	0	0
3 " 50 "	53	74	51	15	4	4	6
4 " 15 "	24	58	62	29	11	10	13

The results are listed in Table X. It may be seen that the retarded eggs behave as well with respect to the type of embryos produced as the early dividing ones. The table also shows that only very few of the eggs that form a normal-looking eight-cell stage develop into normal embryos. It might be claimed that this is due to irregularities in the division of the chromosomes, but such irregularities would probably manifest themselves in abnormal divisions before that time. This point will, however, be checked when a study of the chromosomes of the artificially activated eggs is made.

TABLE X

Embryos Produced by Normal-Looking Eggs Isolated in the Eight-Cell Stage

Type of Egg	Number Isolated	Normal Embryos	Top Swimmers	Bottom Swimmers
Early dividing	57	1	8	30
Late dividing	78	1	5	45

POLARITY OF THE ARTIFICIALLY ACTIVATED EGGS

The abnormal development might also be interpreted on the basis of a disturbance in the polarity of the egg. However, assuming that the indentation marked the pole of the unfertilized egg, it was found that the polarity of the artificially activated eggs remained unchanged. These observations were made by means of the vaseline-slide method. When the eggs are allowed to swell in the dilute sea water, the indentation rounds out, and the germinal vesicle is nearest the surface at the formerly indented region (Fig. 9). Upon return to normal sea water the indentation reappears. The reappearance of the indentation can be followed quite easily (Figs. 39 and 40) with respect to the germinal vesicle and proves to occupy the same position as in the original egg. In the eggs which form polar bodies as a result of the treatment, the indentation soon rounds out and the membrane is elevated (Figs. 21, 22, and 23). The contents of the dissolved germinal vesicle moves towards the pole (as determined by the indentation) and the first polar body later appears at that point (Fig. 24). Fourteen eggs were followed in the manner described and all gave the same results. In the eggs which do not form polar bodies, the indentation persists for some time. Eighteen eggs of that type were followed to the first division and in every case the first cleavage plane passed through the polar axis (as determined by the indentation). However, if the original polarity is retained, the second cleavage plane must also coincide with the polar axis. This was found to be true for all of six eggs followed to the second division. It appears then that the abnormal development cannot be attributed to altered polarity of the egg.

DISCUSSION

In the eggs of *Urechis* the sperm appears to be important for the determination of the plane of bilateral symmetry. The small percentage of normal embryos obtained from the artificially activated eggs may then be interpreted as due to the failure of the processes connected with the establishment of bilaterality to occur in all of the eggs, although they may take place "by chance" in some of the eggs. The production of radially symmetrical embryos from the treated eggs is consistent with such an interpretation. In other eggs with spiral cleavage (*Nereis*, *Cumingia*, and *Chætopterus*) there is evidence for the determination of the plane of bilateral symmetry by the entrance point of the sperm. In such eggs development closely simulating the normal has been shown to be quite difficult to obtain, although the amount of normal-looking cleavage obtained is generally quite considerable.

The view expressed here might presumably be tested by the use of an artificial agent the action of which on the egg is more nearly like that of the sperm. The needle of the puncture method may be taken to be such an agent. Brachet (1911) has studied the relation of the point of puncture to the plane of bilateral symmetry in the egg of the frog. In the frog's egg the gray crescent has been shown to form opposite the entrance point of the sperm, and the plane of bilateral symmetry is determined by the position of the crescent. Brachet found that in the punctured frog's egg the position of the crescent has no definite relation to the point of puncture. In those eggs that develop, the median plane forms in relation to the crescent. This would appear then to be evidence against the idea expressed above, and Brachet interpreted the result to mean that the egg itself has a sort of labile bilaterality. However, he points out that the action of the needle is not quite comparable to that of the sperm. He states (p. 357), "le stylet, si fin qu'il soit, est un instrument grossier, dont l'action est plus brutale et surtout beaucoup plus rapide; si habile que soit l'operateur, le fil de platine ou de verre a atteint le centre de l'œuf en une fraction de seconde. Aussi l'œuf reágit-il en masse, et presque simultanément dans toutes ses parties, tout comme quand il se sent pénétré en même temps par 8 ou 10 spermatozoïdes." He suggests using a very fine needle of one to five micra thickness, and allowing it to take ten to fifteen minutes to reach the center of the upper hemisphere of the egg.

The failure of the eggs that extrude two polar bodies to develop has been noted in one other case of artificial parthenogenesis in annelids and mollusks. Morris (1917) found in *Cumingia* that very few eggs with one polar body or with two polar bodies divide, whereas those with no polar bodies may develop into swimming embryos. This result was confirmed by Heilbrunn (1925). In other cases it has been reported that development may proceed whether one, two or no polar bodies are formed. This has been noted for *Mactra* (Kostanecki, 1904, 1911), *Thalassema* (Lefevre, 1907), *Asterias* (Delage, 1901, 1904; Garbowski, 1903), *Amphitrite* (Scott, 1906), and *Chætopterus* (Allyn, 1912; Lillie, 1906).

In *Urechis*, one of the most interesting features of the eggs which fail to extrude polar bodies is their slow and comparatively abnormal response to the treatment. Those that extrude polar bodies respond to treatment as well as though they had been fertilized. It is surprising then that only the former should develop, since it has been generally held that the degree of success attained by artificial agents depends on the extent to which the initial response of the egg to the treatment resembles its response to the sperm. Just (1922), for example, states, "the highest per cent and normality of cleavage and of plutei result when the membrane separation most closely simulates the separation of the vitelline membrane as a cortical response to insemination." In *Urechis* the eggs which extrude polar bodies have undoubtedly received an optimum treatment, whereas the ones that do not form polar bodies have apparently been incompletely activated. But the former have retained only one fourth of the original chromatin of the egg. The ability of the *Urechis* egg to divide would thus appear to depend upon the amount of chromatin present relative to the cytoplasm. However, it is conceivable that by means of other agents or by additional treatment the eggs that extrude two polar bodies may be made to develop, since in the sea-urchin egg fairly normal plutei have been obtained from eggs which contain the haploid number of chromosomes.

SUMMARY

1. The eggs of *Urechis* may be activated by hypotonic solutions ranging from distilled water to 80 per cent sea water, and trochophores indistinguishable from those produced by fertilized eggs may be obtained.

2. The time of exposure necessary to bring about activation increases with increased concentration of sea water.

3. The activated eggs may extrude two, one or no polar bodies. When the exposure is such as to produce 100 per cent activation,

two polar bodies are extruded on practically all the eggs, but to either side of this optimum exposure the proportion of activated eggs with no polar bodies increases. Eggs with one polar body occur in small numbers, and the single polar body is equal in size to the two polar bodies of the normal egg.

4. Only the eggs with no polar bodies divide and form embryos. Those with two polar bodies or with one polar body may sometimes produce uncleaved swimmers.

5. The eggs which extrude no polar bodies show a very poor response in other respects to the treatment. The eggs which extrude two polar bodies respond to the treatment in very much the same manner as if they had been fertilized. The fact that only the former cleave and develop is inconsistent with the view that for development to be obtained the artificially activated egg must behave like the fertilized egg in its initial response to the treatment.

6. The cleavage of the parthenogenetic egg is quite variable both in form and in time of division.

7. The normal embryos are produced in very small numbers. Even when normal-looking eggs in the eight-cell stage are isolated, less than 2 per cent normal development is obtained.

8. Among the abnormal embryos produced by the parthenogenetically activated eggs, a number of larvæ are found that may be classified in a radially symmetrical group. Such embryos may have the form of hollow blastulæ, early gastrulæ, or late gastrulæ, and their development ends without the appearance of bilateral symmetry.

9. The indentation of the unfertilized egg is found to mark the pole, and with respect to that point the polarity of the artificially activated egg is found to remain unchanged.

10. The first cleavage plane is found to pass through the entrance point of the sperm in 71 per cent of the cases. It may be concluded from this that the sperm is instrumental in determining the plane of bilateral symmetry.

11. It is suggested that in the absence of a localized external agent such as the sperm, it is a matter of chance whether the establishment of bilateral symmetry and other processes associated with the orientation of the embryo may be effected. This would account for the low percentage of normal development and the presence of radially symmetrical embryos.

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OBSERVATIONS ON THE METABOLISM OF *SARCINA LUTEA*. I

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The experiments reported in this communication were designed to provide some information on the dynamics of oxygen diffusion into living cells. It seemed desirable, *a priori*, to perform the observations on very small, spherical cells. To this end, micrococci were the forms chosen. After preliminary experimentation, it was found that a strain of *Sarcina lutea* which had long been maintained on artificial culture media fulfilled many of the requirements of the experiments: the organisms are very small and discrete (this strain no longer forms packets); the cells are spherical and highly uniform in diameter; and in water or dilute aqueous solutions they remain sufficiently viable for the requisite periods of time under the conditions used. As the experiments progressed there were noted a number of interesting characteristics of their metabolism, which are the main subjects of this paper.

METHODS

1. *Bacterial Cultures.*—(*a*) Preparation of suspensions: The organisms grow luxuriantly on plain nutrient agar prepared with peptone and fresh meat infusion or beef extract, with or without the addition of NaCl. After the completion of the preliminary experiments, the medium used contained one per cent Difco peptone, 0.35 per cent beef extract and 1.5 per cent agar in tap water. Agar slant cultures in tubes or Kolle flasks were grown for 20–24 hours at 37° C. The growth was washed off with distilled water, the suspension homogenized by gentle but prolonged shaking, the microörganisms precipitated by centrifugation and then washed by two suspensions in and

precipitation from distilled water. After the final washing, the sedimented cells were again suspended in distilled water and shaken to give a concentrated homogeneous suspension.

(*b*) Enumeration of cells and pH measurements: The numbers of bacteria per cc. were determined before and after exposure to the specific conditions of the experiments by direct microscopical observation and by plate count. The former was performed by the hemocytometer method, using a dilute solution of carbol fuchsin as a diluting fluid; the latter as commonly used in the enumeration of bacteria in water, milk and other fluids. The direct count includes all cells visible under the microscope and the plate count only viable cells which developed on the medium used into colonies visible to the eye aided by a 5–10 × hand lens. Although all reasonable efforts were made to insure accuracy, the usual presumptive errors of these methods apply to our data.

The suspensions used in our experiments were, before dilution, thick, yellow, pasty emulsions of the sedimented, packed cells. Microscopical observations indicate a high degree of freedom from detritus. The concordance of the direct and plate counts made before and after performance of the experiments implies a high coefficient of vitality and the essential absence of dead or senile cells in the suspensions when first prepared.

Measurements of hydrogen ion concentration were performed with indicators by a spot plate method. Inasmuch as the suspensions consist of the bacterial cells in distilled water or dilute aqueous solutions of low intrinsic buffering power, the stability of the pH of an exposed suspension is controlled primarily by the buffering powers of the cells. From preliminary titration experiments (after the method of Shaughnessy and Falk, 1924) it developed that the strain of *Sarcina lutea* used displayed rather slight buffering power. Hence, in performing colorimetric pH measurements, we determined with which of a series of solutions of very low buffer action, and varying but known pH values, the test solution was isohydric. The standard solutions were prepared by adding twice the usual concentration of indicator solution to water and adjusting the pH of 10 cc. samples to various levels (at 0.2 pH intervals) by adding appropriate quantities of HCl or NaOH (*cf.* Fawcett and Acree, 1929). The colorimetric standards were checked by electrometric measurements, using a buffer solution standard and the quinhydrone electrode. The results of several experiments appear in Table I.

It will be noted that the direct and plate counts generally agree closely at the outset of an experiment and are not too widely apart at

TABLE I

Hydrogen Ion Concentrations and Microbic Enumerations for Suspensions of Sarcina Lutea

Date 1929	Suspension	pH	Number of cocci per cc. ($\times 10^{-8}$)	
			Direct	Plate
Apr. 27	Original		3.2	1.7
	After 2 hours (room temperature):			
	Original		2.6	2.1
May 30	Original	7.4	143	140
	After 6 hours:			
	In water shaken in air	6.8	107	430
June 6	Original	7.4	63	64
	After 6 hours:			
	Original	7.2	150	30
	In glucose shaken in air	?	150	30
	In water shaken in N_2	7.6	32	27
	In glucose shaken in N_2	7.5	41	27
	In water shaken in air	8.0	40	7
July 5	Original		70	26
	After 6 hours:			
	Original		102	29
	In water shaken in air		23	9
	In glucose shaken in air		27	11
	In glucose shaken in 1% O_2		13	14
	In water shaken in 1% O_2		58	13
Aug. 7	Original		85	13
	After 11 hours:			
	Original	7.4	71	17
	In water shaken in air	7.8	23	8
	In glucose shaken in air	7.2	8	6
	In water shaken in N_2	7.2	6	8
	In glucose shaken in N_2	6.4	14	6
Oct. 16	Original	7.4	199	55
	After 26 hours:			
	In water shaken in N_2	7.8	182	29
	In glucose shaken in N_2	6.4	202	180
	In glucose shaken in air	6.0	139	35
	In water shaken in N_2	7.8	170	35
	In water shaken in air	7.6	148	32

the completion. When variations occur they are generally, as is to be anticipated, in the direction of lower plate than direct counts.

(*c*) Size of the bacteria: The diameters of the bacterial cells were determined by direct microscopical measurements with hanging drop preparations and a filar micrometer. There were no significant variations in the size of cells suspended in water and in dilute glucose suspensions, with or without traces of dilute, neutral fuchsin. The average diameter determined from several hundred measurements on the cocci was 1.275 micra. In this series the maximum was 1.42, the minimum 1.08. The distribution was approximately symmetrical about the mean and the probable error of the mean was $\pm 0.07 \mu$.

(*d*) Final handling: The thick bacterial paste obtained after centrifugation was diluted 1 : 1 with distilled water or with 1.0 or 0.5 per cent glucose in water and well mixed by shaking (with a glass bead). Of these suspensions 0.4 cc. (dry weight about 5 mgm., containing about 2×10^9 bacteria) or 0.8 cc. portions were placed in the manometer vessels for determination of oxygen consumption, etc., and a similar amount dried on a cover slip and weighed. In certain experiments oxygen was bubbled through the suspension for some time before beginning the observations. The pH was usually determined before and after the manipulation by the method described above.

2. *Gases.*—Tank nitrogen, of 99.7 per cent purity, was passed over heated copper and through glass connections directly into the manometers. Even this treatment did not give absolute freedom of oxygen, but traces that remained did not affect the results (see below). Gas mixtures containing 0.5 to 10 per cent oxygen were made by volumetric mixing of the treated nitrogen and air in large bottles over water. The nitrogen was always bubbled through first for a considerable time to remove dissolved air, and the mixture was used at once after preparation. To obtain accurate mixtures of 0.5 per cent or less, a manometric method was used. This is described below.

3. *Measurement of Oxygen Consumption, Carbon Dioxide Production, etc.*—The Warburg methods were used throughout. Small chambers of the cylindrical or conical type with an inset, used for studying metabolism of nerve (Gerard, 1927), were found highly satisfactory. With 0.4 cc. of bacterial suspension and 0.2 cc. of $N/10$ NaOH in the inset to absorb CO_2, the constants were either about 0.25 or 0.35, so that one cu. mm. change in gas volume gave a pressure difference of 3 or 4 mm. (of Brodie's solution). Oxygen consumption, Q_{O_2}, is expressed as cu. mm. consumed per hour per mgm. dry weight of the bacteria.

The respiratory quotient (R.Q.) was determined by comparison of the manometer readings when CO_2 was absorbed (NaOH in inset) and when allowed to accumulate (water in inset) in parallel experiments. Acid production was determined manometrically by the liberation of CO_2 from a bicarbonate-Ringer solution in the presence of 5 per cent CO_2 in nitrogen (Warburg, 1926).

All experiments were carried out at 20.1° C.

Gas mixtures with low concentrations of oxygen were obtained by mixing in a manometer. The Brodie solution used in the manometer capillary has a specific gravity so adjusted that 10,000 mm. equals 760 mm. of mercury. The pressure of the gas in the manometer chamber is easily obtained from the barometer reading and the difference of fluid level in the two limbs of the manometer. It is usually sufficiently accurate, in determining the percentage of oxygen, to assume 10,000 mm. Even the maximal correction for water vapor, if dry air is admitted, is only 5 per cent and may ordinarily be omitted. The oxygen admitted to the chamber filled with nitrogen is determined as follows: the fluid levels in the manometer are lowered (by a screw at the bottom) until there is a "negative" pressure in the chamber of *circa* 100 mm. (left side open to air = 50 mm., right side to chamber = 150 mm.). The chamber is then opened to the air for an instant by rapidly turning a stop-cock, the apparatus is shaken for a moment, the fluid level on the chamber side is brought to its initial setting and the level in the open limb read. This will now be, say, 200 mm. At constant volume and temperature then (the room air being nearly at the temperature of the thermostat and coming to rapid equilibrium) the gas pressure has been increased by 150 mm. of air or 30 mm. of oxygen. With a little practice it is possible to adjust the initial pressure difference and time of opening the chamber so that any desired oxygen percentage up to 0.5 can be obtained by one operation. This method permits the rapid and accurate preparation of gas mixtures with very low oxygen concentration and should be valuable in other studies where the influence of oxygen pressure on oxygen consumption is to be determined.

RESULTS

1. *Respiration in Air.*—The rate of oxygen consumption for any sample of the bacteria used is not constant with time, but tends to be high at first and to fall rapidly at the outset and then slowly. Sometimes it remains at a constant low level from the third or fourth hour on; more often a slow fall continues through a 20-hour period. A typical curve is shown in Fig. 1, and the results of several experiments are given in Table II.

The initial level of oxygen consumption and the amount of fall have been fairly constant from one test suspension to another and variations are not related to variations in the ages of the cultures used. It might be anticipated that bacteria taken from a culture which has passed the period of logarithmic rate of growth would show an approximately uniform level of oxygen consumption. Although viability generally decreases in the non-nutrient menstrua, the rate of this process is far too low to be invoked as the explanation of the decline in oxygen consumption. The question arises: In what way does the preparation of the suspension and its treatment in the manometer chamber lead to the establishment of a "time zero"? In other words, if the oxygen consumption of these cocci is assumed to have been essentially constant in time, what causes it to begin a rapid fall when its measurement is undertaken?

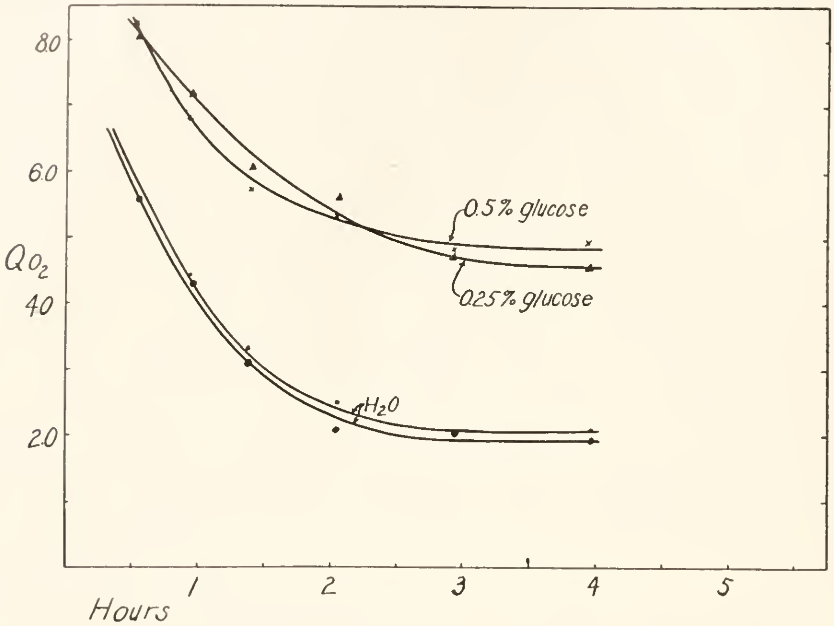

FIG. 1.

If a progressing mortality of the individual cells does not seem to explain the findings, the early high respiration cannot, on the same evidence (some variable fall in plate counts from start to end), be interpreted as due to continued growth which was later suspended. Continued agitation, gentle or vigorous, has been shown to have no effect; and temperature and suspension menstruum can also be

TABLE II

Date 1929	Condition	Q_{O_2} of *Sarcina lutea* Hours after start of experiment					
		1	2	3	4	5	6
May 14	Water suspension, in air	6.0	3.5	2.3		1.6	
	Water suspension, in air	6.5	3.5	2.3		1.7	
	0.2% glucose suspension, in air		5.5	3.8		2.4	
	0.2% glucose suspension, in air		5.5	3.4		2.2	
May 30	Water suspension, in O_2	4.4	2.2		2.0		
	Water suspension, in air	4.3	2.0		2.0		
	0.5% glucose suspension, in air	6.9	5.1		5.0		
	0.2% glucose suspension, in air	7.1	5.2		4.6		
June 6	Water suspension, in air		6.8	5.2	4.0	3.3	2.8
	Water suspension, in 1% O_2		2.9	2.3	2.0	1.8	1.6
	0.2% glucose suspension, in 1% O_2		5.0	3.7	3.0	2.6	2.3
July 3	Water suspension, in 2.5% O_2	5.2	2.7	1.9	2.1	1.7	
	Water suspension, in 1% O_2	6.0	2.7	2.1	2.2	1.7	
	0.2% glucose suspension, in air	7.2	5.2	4.5	4.4	3.7	
	0.2% glucose suspension, in 2.5% O_2	11.0	5.1	4.0	3.7	3.1	

TABLE II—*Continued*

Date 1929	Condition	Q_{O_2} of *Sarcina lutea* Hours after start of experiment					
		1	2	3	4	5	6
Aug. 7	Water suspension, in air	8.6		← 4.1 →			
	Water suspension, in air after 4 hours anoxia	14.4		6.2			
	Water suspension, in air after 4 hours anoxia	11.8		6.0			
	0.2% glucose suspension, in air	21.1		11.6			
	0.2% glucose suspension, in air after 4 hours anoxia	34.8		19.0			
	0.2% glucose suspension, in air after 4 hours anoxia	31.8		16.6			
Oct. 16	Water suspension, in air	3.2	2.1	1.8		←1.2→	1.1 (9 hrs.)
	Water suspension, in air after 1 hour N_2 bubbling	3.3		← 1.7→		←	1.1→(9hrs.)
	Water suspension, in air after 1 hour O_2 bubbling	1.8		← 1.4→		←	1.1→(9hrs.)
	Water suspension, in air after 13 hours anoxia	9.1		2.1			
	0.2% glucose suspension, in air after 1 hour anoxia	7.5		← 3.8→		←	2.1→(9hrs).
	0.2% glucose suspension, in air after 13 hours anoxia	12.2		4.6			
Nov. 14	Water suspension, in air	4.3	←3.1→		←1.7→		←1.3→(9hrs.)
	Same	4.3	←2.5→		←1.7→		←1.3→(9hrs.)
	0.2% glucose suspension, in air	9.3	←3.3→		←1.8→		←1.6→(9hrs.)
	Same	8.8	←4.5→		←2.0→		←1.6→(9hrs.)

excluded as factors leading to the fall in oxygen consumption. It might then be assumed that the initial values are excessive, probably largely as a result of partial asphyxia of the organisms induced in the course of their preparation (and partly perhaps due to food reserves stored from the nutrient medium), and that the lower values obtained some time after an experiment is begun are to be regarded as the "normal" ones rather than as "abnormal" levels resulting from some depression. It may be noted that Callow (1924) obtained a Q_{O_2} of 7.0, constant for 10 hours, using *Sarcina aurantiaca.*

When the organisms are suspended in a 0.2–0.5 per cent glucose solution instead of water, the same progressive fall of oxygen consumption is observed. The Q_{O_2} values at each point in time are, however, much greater in the former case (by 100 per cent to 300 per cent), so that the respiration curve for the bacteria in the glucose-containing menstruum lies above but parallel to that for the bacteria in water. (Fig. 1, Table II.) The influences of glucose and other substances on the respiration of *Sarcina lutea* are reported upon in more detail in the following paper.

The R.Q. in water and in glucose solution was determined in two experiments. In water it remained constant at about 0.67 during 8 hours. (Gotschlich, 1912, cited quotients of 0.71–0.78 for bacteria metabolizing in the absence of fermentable substances, and Stephenson and Whetham, 1923, found R.Q.'s of about 0.7 after all added glucose had been utilized.) In glucose solution, the R.Q. for the first hour was 0.95 and then fell during seven hours to 0.71, the average for the whole run being 0.81. These figures suggest that, even in glucose solution, less than half the oxygen consumed is used to oxidize carbohydrate, although the presence of glucose has more than doubled the amount of oxygen taken up. (These values of R.Q. are lower than those recorded for bacteria on a nutrient pabulum capable of supporting their growth. *Vide* Soule, 1928.)

2. *The Influence of Oxygen Pressure.*—The oxygen consumption of *Sarcina lutea* in equilibrium with oxygen at varying partial pressures has been determined and it has been found that in air or pure oxygen the respiration is essentially the same. When the bacteria were held in water, with a lower rate of respiration, 2.5 per cent oxygen in the gas or even one per cent oxygen, in two cases, sufficed as well as the pure gas. With the glucose menstruum (and more rapid oxygen consumption) one per cent oxygen was definitely too low a concentration to support full respiration and 2.5 per cent of the gas was sometimes inadequate. Concentrations below one per cent were uniformly insufficient to support the rate of oxygen consumption possible in air.

When, after a period of complete anoxia, oxygen was admitted to a concentration of 0.5 per cent or less, there was at once a moderate oxygen consumption. Although the rate of respiration was already far less than in air, it fell off considerably in a short time, much as previously described for experiments in air (Fig. 1). The fall in this case was not due to a diminishing supply of oxygen following utilization of the small amount admitted, for it occurred when only 5 to 10 per cent of the oxygen had disappeared. More than doubling the amount of shaking did not affect the value of the critical oxygen pressure That failure to attain equilibrium between gas and liquid phases did not play a rôle is further attested by experiments made in another connection. Hydrogen was absorbed by an unsaturated fat in water suspension under the influence of a catalyst at a rate over 100 times that of the oxygen absorption here, yet the rate of shaking was adequate.

3. *Oxygen Debt.*—It seemed possible that the regular occurrence of a relatively high oxygen consumption directly after admitting this gas, or at the start of an experiment as the bacterial suspensions became well oxygenated by shaking, might represent the discharge of an oxygen debt. That such a debt can be accumulated is easily demonstrated: a suspension kept for some hours in nitrogen and then exposed to air takes up oxygen at a greatly increased rate (Fig. 2). At the start, this rate may be considerably greater than observed when the bacteria are kept in air throughout. It subsequently

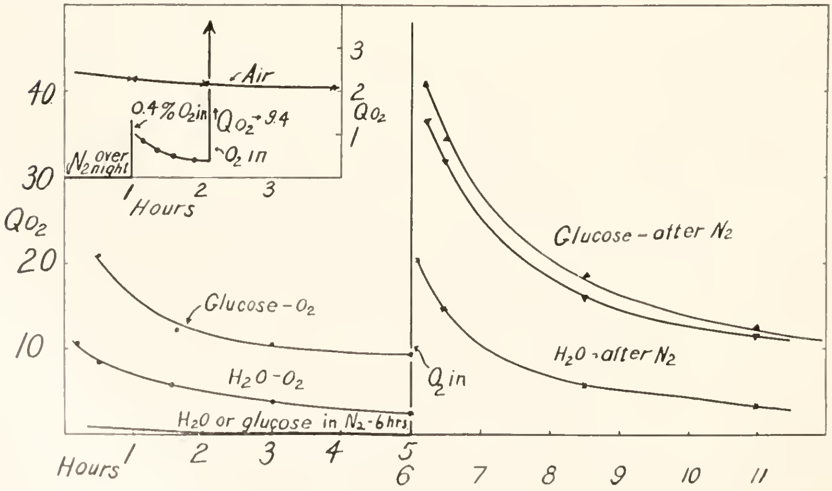

Fig. 2.

decreases just as under the conditions previously considered. This burst of increased oxygen consumption is greater after long than after short periods of anoxia, and for the same period is greater when the bacteria are suspended in glucose solution than in water (Table II). Glucose is capable, apparently, of stimulating the metabolism of these organisms not only in the presence of oxygen (leading to a more rapid consumption of the gas), but also in its absence (leading to an increased oxygen debt).

To determine whether previous anoxia was the cause of the high respiration observed at the start of the simple experiment in air, the following test was made. A particular bacterial suspension was divided into two portions. Oxygen was bubbled through one and nitrogen through the other for an hour and then each was rapidly introduced into manometers in air. The usual high initial oxygen consumption values were obtained with the sample treated with nitrogen, while they were much lower for the oxygen-treated portion. The slow fall, continuing for many hours after mounting, occurred with both. This slow fall of oxygen consumption with time might be related to the exhaustion of the substrate to be oxidized rather than to the oxygen itself. In the case of water suspensions, at least, the only material for oxidation that the cells have is their own substance and such food as may be stored during growth in a nutrient medium. During 24 hours the carbon in the carbon dioxide given off corresponds to 1 to 2 per cent of the dry weight of the organisms.

4. *Acid Formation.*—The oxygen debt these cells incur in an atmosphere of nitrogen might be due to accumulation of oxidizable metabolites or to exhaustion of an oxidizing reserve. The first case is reminiscent of the metabolism of muscle and the rôle played by lactic acid, the second suggests the metabolism of nerve. We therefore attempted to ascertain whether acid is produced in the metabolism of the microbic cells under the conditions of our experiments—especially in the absence of oxygen.

The pH of the cell suspension was determined in many experiments before and after manipulation in the manometers, using the indicator method described above. There was no consistent change under any of the conditions studied—water or glucose solution, oxygen present or absent. The initial and final pH values were about 7.4, though occasional final values were as high as 8.0 and as low as 6.0, especially in glucose suspensions. Acid formation was also measured manometrically by the liberation of CO_2 from bicarbonate-Ringer solution in equilibrium with 5 per cent CO_2 in N_2. This was studied in the presence or absence of glucose. During eight hours' asphyxia, the

CO_2 liberated, if due to lactic acid formation, indicated a production of 0.0023 mgm. of lactic acid per mgm. dry weight per hour in glucose solution and 0.0001 mgm. lactic acid in water (one experiment each). The quantities of lactic acid formed in eight hours would require, respectively, 14.5 and 0.6 cu. mm. of O_2 for complete oxidation. For many cells, including bacteria, studied by Meyerhof (1927) (also: Meyerhof and Finkle, 1925) and others, the oxidation quotient (the lactic acid produced in nitrogen minus that formed in oxygen, divided by the oxygen consumed) is 3 or more. That is, one equivalent of oxygen consumed prevents the appearance of three times as many equivalents of lactic acid as it could oxidize. In these experiments, in eight hours' exposure in an oxygen atmosphere, the bacteria consumed: in glucose solution, 24 cu. mm., and in water, 17.5 cu. mm., of oxygen. The oxidation quotients are, therefore, 0.6 and 0.03. The lactic acid (?) produced during asphyxia was only one-fifth of what might have been anticipated in the glucose solution and was practically zero in the water.

These results may be expressed in another way. In other experiments, in either water or glucose, following a six-hour asphyxia the average extra oxygen taken up during 6 hours was 50 per cent of the normal oxygen consumed by the same number of bacteria in the same time. The total oxygen debt in these experiments may therefore be assumed to have been 9 cu. mm. in glucose solution and 5 cu. mm. in water. Since in the water suspension only 0.6 cu. mm. of oxygen could have been used to oxidize acid metabolites (typified by lactic acid), nine-tenths or more must have acted in some other manner. In glucose solution, the extra oxygen might have been used entirely to oxidize metabolites, though presumably here also some would be used otherwise. The possibility must be recognized, of course, that more acid may have been formed than is recorded by the CO_2 liberation, some being neutralized by other cell buffers. Also, intermediate substances of too weak acidity to result in the liberation of CO_2 would escape detection by the methods used.

5. *Oxidizing Reserve.*—The existence of a large oxygen debt after asphyxiation, with no demonstrable accumulation of incompletely oxidized metabolites to account for it, suggests that the extra oxygen is utilized to replenish an oxidizing reserve which had been drawn upon to continue oxidations during asphyxia. If this were the case, some CO_2 should appear during the asphyxial period and a correspondingly smaller amount during the following oxygen period. The R.Q. of the extra respiration, while the oxygen store is being refilled, should be correspondingly low. This does not appear to be the case,

for the R.Q. of suspensions in water is the same early or later after commencing a series of observations, whether or not the extra oxygen consumption is occurring. Also, the absence of CO_2 liberation in nitrogen from water-bicarbonate suspensions renders improbable the production of CO_2 as well as of non-volatile acids. We do not wish, on the basis of the few experiments performed, to conclude finally that the oxygen debt developed by *Sarcina lutea* is due to the accumulation of non-acid metabolites and not to the depletion of oxidizing reserves, though this seems most probable. The work unfortunately could not be continued at the time and the present report must suffice to record these preliminary observations. It is interesting to note that Meyerhof (1912) found in some of his studies on *Vibrio metchnikovi* that in the absence of oxygen all chemical activity and energy liberation appeared to be simply suspended. The falling Q_{O_2} with time observed for *Sarcina*, in contrast to the constant respiration of bacteria observed by Callow (1924), may be related to such differences.

SUMMARY

The respiration of a strain of *Sarcina lutea*, growing as individual cocci of uniform size and of relatively high viability under our experimental conditions, was studied by means of the Warburg technique. Suspensions of thoroughly washed bacteria in water or in glucose solution were enumerated by direct and plate counts, and pH determinations were made before and after manipulation in the manometer. A simple method is described for obtaining accurate gas mixtures with a low concentration of oxygen.

The oxygen consumption of various suspensions of the washed cocci was found to be fairly constant in water suspension at about 2.6 cu. mm. O_2 per mgm. dry weight per hour, or approximately 7 μ^3 O_2 per single cell per hour: that is, over three times its volume of oxygen was consumed per hour by each micrococcus. This value is for the nearly constant level attained some hours after the start of the experiment. Oxygen consumption is considerably more rapid at first and falls along a roughly arithlogarithmic curve towards an asymptote. This early excess appears to represent an oxygen debt due to partial asphyxia produced in the course of preparation of the suspensions.

In 0.2 to 0.5 per cent glucose solution, the rate of oxygen consumption falls along a similar curve, but the rates are 100 to 300 per cent greater than for suspensions of cocci in water.

The respiratory quotient in water is constant at about 0.67; in glucose it falls from 0.95 to 0.71 in seven hours (one experiment each).

The oxygen consumed by a suspension in water is independent of oxygen concentration when this is above one per cent. For glucose suspensions 2.5 per cent O_2 or more is required for maximal consumption. Below the critical values of oxygen tension, oxygen consumption becomes less with diminishing oxygen concentration. When air is admitted to the suspension after a period of complete anoxia, there is observed a high initial rate of consumption followed by progressively declining rates during several hours until a relatively constant rate is approached. When small amounts of oxygen (0.5 per cent or less) are admitted after anoxia, consumption is greater at first and then falls as when air is admitted; but the maximal rate is low, usually less than the normal in air.

The oxygen debt developed by these organisms is not apparently associated with the production of detectable acid metabolites (in water suspensions, at least), nor with the formation of carbon dioxide during asphyxia. It may result from the accumulation of non-acid metabolites, or of amounts of acid metabolites that do not equal or exceed the buffering capacities of the cells. The oxidation quotient, even in glucose solution, in contrast to the data for most cells that have been studied, is not over 0.6.

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OBSERVATIONS ON THE METABOLISM OF *SARCINA LUTEA*. II

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In the preceding paper the effects of anærobiosis on this organism and the influence of glucose on its oxygen consumption have been reported. It seemed desirable to extend these observations to the influence of lactic acid, as one of the carbohydrate intermediates, and also to study the influence of the respiratory catalyst, methylene blue, and of the inhibitors, carbon monoxide and cyanide. The results of these experiments have been surprising in several respects. The methods used were exactly as previously described, and all experiments were carried out at 22° C.

Results

1. *Water and Saline Suspensions.*—This series of 34 experiments in water shows an average Q_{O_2} of 2.5 during the relatively steady period five hours after the start of readings (Table I). This is in good agreement with the value of 2.6 for the first, smaller series. The temperature was slightly higher in this series but the average was

Table I

Q_{O_2} *in Water Suspension*

Initial value Q_{O_2}	Later value	
	Hours	Q_{O_2}
8.1 (4) *	5	3.5
9.3 (4)	5	3.3
7.2 (4)	4.5	2.4
7.5 (4)	4 +	2.5
8.5 (2)	4.5	1.8
5.3 (4)	5	2.1
5.8 (2)	4.5	2.0
5.1 (3)	3.5	2.2
4.7 (2)	6	1.2
6.1 (1)	5.5	3.0
8.0 (4)	5	2.3
? 1.4 (2)	5	0.8
? 1.8 (3)	3	1.2

* Bracketed number represents number of separate runs.

taken at a somewhat later time. The initial Q_{O_2} average is 7.1, a value depending on the conditions of preparation, as previously discussed. Two experiments at 37° C. compared with 22° C. gave a Q_{10} of approximately 1.7.

Barron and Harrop (1929) found a marked effect of crowding on the Q_{O_2} of white blood cells, the respiration per unit of material falling as more and more concentrated suspensions were used. The possibility of a similar effect in these experiments was tested by varying the density of the suspension from the start or by suddenly diluting a water suspension during a run by tipping in more water. No change was observed, so that this factor may be excluded. It might well depend, in the case of the blood cells, on limitation of motility of the undulating membrane in the heavier suspensions.

The effect of NaCl in concentrations up to 1.1 per cent was determined, as a control of osmotic and ion effects with other reagents. The lower concentrations were without effect, and even the highest used gave a maximum decrease of 20 per cent of the Q_{O_2} as compared with water, and this only occasionally. Further addition of one part to ten of M/15 phosphate buffer was likewise of no consequence for the oxygen consumption; nor was the pH, between 7 and 8. When salt solutions were tipped into a water suspension of respiring bacteria, a small brief acceleration was often observed, after which the original curve was resumed (Fig. 3). Whether this is a true momentary "stimulation" or some small experimental error has not been further investigated; in either case the results serve as a control for the observed changes when other substances are tipped in.

2. *Methylene Blue.*—Addition of methylene blue (0.2 per cent or 0.1 per cent methylene blue "for vital staining") to a water or buffered saline suspension always raised the Q_{O_2} by about one hundred per cent; from an average value of 1.9 to one of 3.9 in seven experiments, an increase of two. The increased oxygen consumption tends to fall off with time but remains above that of water for several hours. The effect of methylene blue plus glucose, sodium lactate, or sodium cyanide will be considered with those substances.

3. *Glucose.*—The effect of glucose addition has been followed somewhat further than in the preceding paper, by tipping a glucose solution into a water suspension of the cocci during a run. This makes it possible to obtain the entire glucose effect, since no time is lost after its addition, during which readings cannot be made. Table II sums up all the results with plain glucose. The average Q_{O_2} of 2.2 before tipping in the sugar rose to 10.5 for the first period (one-half to one hour) after tipping and then fell. At three and one-half

TABLE II

Respiration in water before addition	Added		Maximum after addition	Later value	
	Glucose	Methylene blue			
Q_{O_2}	Per cent		Q_{O_2}	Hours	Q_{O_2}
* 2.3	.5	+	13.6	6	1.3
2.5	.5	–	10.4	6	4.1
2.1	8.8	–	15.0	5	4.5
2.0	0.4	–	9.7	4.5	4.5
1.9	0.2	–	9.3	2	8.5
2.5	0.8	+	14.6	2	14.4
2.3	0.8	–	9.2	2	9.7
2.2	0.2	+	19.2	2 (18)	4.9 (0.5)
2.3	0.2	–	10.0	2 (18)	4.4 (1.2)
2.3	0.8	+	18.2	2 (18)	13.2 (0.3)
2.3	0.8	–	12.2	2 (18)	8.2 (0.5)

* Parallel runs.

hours the average Q_{O_2} was six. The maximum reached was independent of glucose concentration (though 8.8 per cent glucose gave the greatest increase observed), but the subsequent fall was somewhat slower with larger amounts than smaller.

The total excess oxygen consumed by the bacteria due to the addition of glucose can be determined by subtracting the Q_{O_2} curve in water from that with glucose added. In at least two cases this has been definitely greater than the amount required to completely oxidize all the added glucose, which suggests a "specific dynamic action" of this substance. Confirmatory evidence is obtained from the R.Q. findings, discussed previously, and from the further observation that suspensions carried for 24 hours in glucose solutions have a lower Q_{O_2} than similar ones in water, as if the higher rates at first had more completely exhausted the cell reserves. Evidence of a stimulating action of glucose in nitrogen has also been previously presented. All these observations indicate a dynamic action of glucose though they are hardly extensive enough to permit sweeping conclusions.

Methylene blue plus glucose leads to a greater initial increase of oxygen consumption than the sum of their separate effects, but this excess becomes less with time (Fig. 1). Thus, in four parallel experiments, the average maximum in glucose was 11.2, in glucose plus 0.2 per cent methylene blue, 16.5. Seven hours later the values were 4.7 and 5.8, and the next morning, in all cases followed, the values were higher in the suspensions in glucose without the dye. This diminishing or reversing of the methylene blue increase with time is similar to the results with water solutions of the dye or of glucose alone. The absolute increase of Q_{O_2} when methylene blue is added to water suspensions is 2.0, but the percentage increase is about one hundred. When the dye is added to glucose suspensions, the absolute increase is over 5, the percentage increase fifty. The interpretation comes to mind that these two substances act at successive steps in the chain of oxidative reactions, thus leading to a product of their separate effects, rather than concomitantly, which should give only the sum. Much evidence exists that this is indeed the situation. The recent findings of Warburg, Kubowitz and Christian (1930) and Wendel (1930) indicate that reduction of methylene blue may lead to peroxide formation with cytochrome, which in turn oxidizes other substances (as lactic acid to pyruvic acid), and leaves the iron in the Fe^{+++} stage. The second reaction might well depend, if only indirectly, on the amount of oxidizable material (glucose) present. The findings with lactate additions, however, are not entirely in harmony with this view.

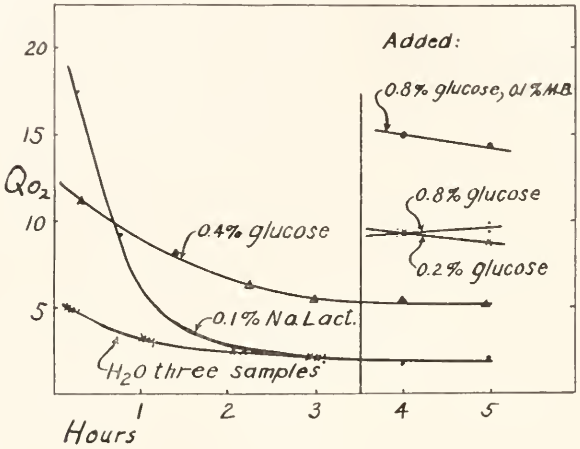

Fig. 1.

4. *Sodium Lactate.*—The greatest rates of oxygen consumption obtained resulted from the addition of lactate to water or saline suspensions. The maximum Q_{O_2} after tipping averaged over ten times that just before, and a more than twenty-fold increase has been observed. Though the maximum Q_{O_2} varied in different runs from

17 to over 50, the variation was low in any one run and not related to the concentration of lactate added (Fig. 2). (In one experiment a regular rise in maximum with concentration was observed.) The subsequent course of the extra oxygen consumption, on the other hand, was intimately dependent on this concentration (Figs. 2 and 3, Table III). Thus, the average of a series of twelve experiments gave a Q_{O_2} of 2 in water, and a maximum of 23 after sodium lactate was tipped. Five hours later the Q_{O_2} values for various concentrations were:

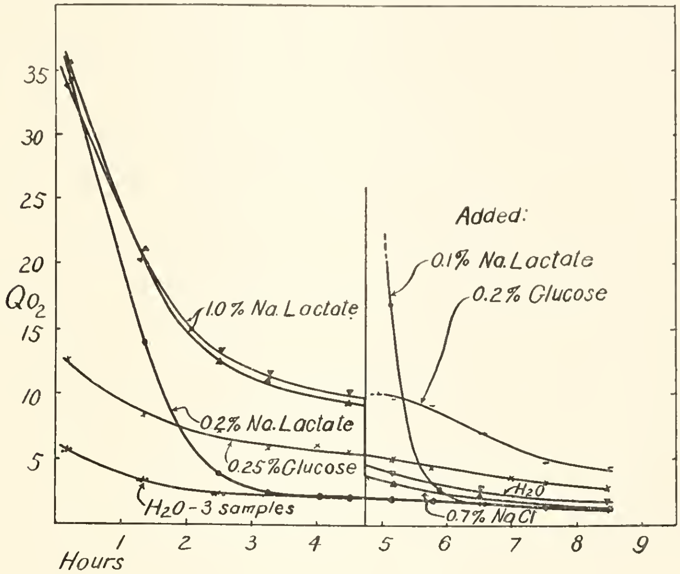

FIG. 2.

0.05% = 1.4; 0.1% = 2.5; 0.25% = 3.0; 0.55% = 6.0; 1.1% = 7.0.

Such a falling off with time, of the lactate effect, is due in part to the actual removal of lactate, but cannot be accounted for entirely on this basis. A few hours after the addition of 1.0 per cent lactate ions, the Q_{O_2} will have fallen to, say, one-half of the maximum. It can be inferred (see below) with considerable certainty that over 0.5 per cent lactate is still present. It has been found, however, that when 0.5 per cent lactate is freshly added to a water suspension, the Q_{O_2} reaches the same maximum as when 1.0 per cent is added; so that the falling off in the first case must be complicated by other factors. An occasional finding, which also indicates the complexity of the system, is an immediate increase of respiration rate after lactate (or glucose) addition followed by a further rise for an hour or more before the usual fall sets in. This appeared oftener with the more concentrated additions, as if the strong lactate partly inhibited respiration until some was metabolized away.

The amount of lactic acid actually burned may be estimated from the excess oxygen consumed, on the assumption that just this extra oxygen is all used to completely oxidize the acid. In most experiments 0.2 cc. of a 0.2 per cent solution of lactate (as the sodium salt) was tipped into 0.2 cc. of the bacterial suspension, giving a final concentration of 0.1 per cent. This quantity of lactic acid, 0.0044 millimols, would require 298 cu. mm. of oxygen for full oxidization. The values actually obtained for the extra oxygen consumed (the area under the Q_{O_2} curve with lactate present less that with lactate absent —the two curves usually joining some hours after the start) were:

TABLE III

Respiration in water before addition	Added		Maximum after addition	Later value	
	Sodium lactate	Other substance			
Q_{O_2}	Per cent		Q_{O_2}	Hours	Q_{O_2}
3.5	.2	Methylene blue	48	7	1.5
4.3	.2	Methylene blue	52	7	0.5
4.5	.2	—	65	7	3.0
2.6	.2	Methylene blue	17	6	1.1
2.5	.2	—	20	6	1.9
1.8	2.2	In water	17	4.5	8.3
	1.1	In NaCl, PO_4 buffer, pH7	18	4.5	7.4
1.4	1.4	Same, pH8	19	4.5	8.2
2.2	2.2	—	29	5	5.5
2.0	0.5	—	28	5	3.0

210, 250, 200, 270, 310.

The average, 250, is somewhat less (15 per cent) than theoretical for the complete burning of all the lactate. This is probably a technical error due to failure to rinse all the fluid from the side bulb into the main chamber, with the consequent exclusion of some of the lactate from the bacteria. Unfortunately, the quantitative possibilities were not in mind when the experiments were carried out, and the usual to and fro pouring was ordinarily omitted because of the danger of spilling alkali—a narrow opening to the side-bulb made rather severe tapping necessary in some cases. In general, the agreement is good enough to indicate complete oxidation of small amounts of lactic acid.

With larger quantities, that is, more concentrated solutions, oxidation was not complete even in twenty-four hours—when the extra oxygen consumption had ended. Thus for a 1.0 per cent solution 1500 cu. mm. of oxygen would have been required for full oxidation. The excess oxygen totalled over 700 cu. mm., and the total oxygen used (*i.e.*, the lactate Q_{O_2} curve not corrected by the water control) after the lactate addition only about 900 cubic millimeters.

The possibility at once suggests itself that the falling off of the excess respiration, and the failure of complete oxidation of the larger amounts of lactic acid are alike due to the accumulation of injurious reaction products. A rise in pH, in particular, must result from the conversion of lactate ion into CO_2, two-thirds of which would leave the bacterial suspension and be absorbed in strong alkali in the inset of the vessel, while one-third would remain to combine with the free Na^+ ions, giving $NaHCO_3$ in place of $NaC_3H_5O_3$. Such a pH change is not, however, a critical factor; for suspensions buffered at pH 7 or 8 show the same Q_{O_2} curve with added buffered sodium lactate as do water suspensions (Fig. 3, Table III). Also, in one series, a phosphate buffer at pH 5.9 was added to the suspensions in 1/10 and 1/5 concentrations. After a long run with much added lactate, the 1/10 buffer was exhausted and the suspension alkaline to litmus, while that with the stronger buffer was still faintly acid. The Q_{O_2} curves, however, were almost perfect duplicates. Further, it is doubtful if any kind of inhibiting end-products can be involved. If such were the case,

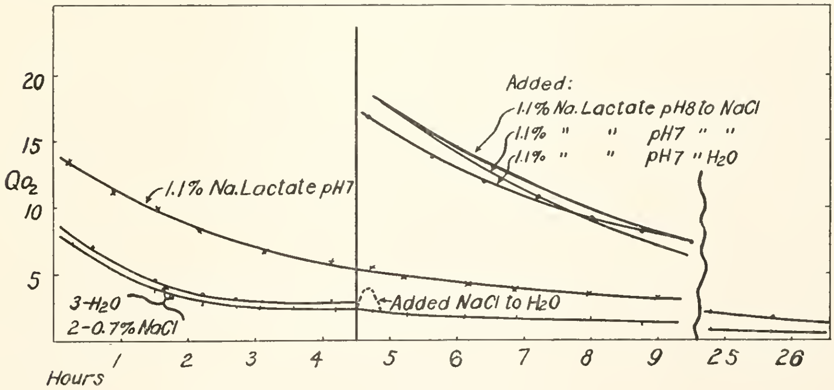

FIG. 3.

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dilution of a suspension which has had lactate added some time before should cause the fallen Q_{O_2} to again rise to some extent. Actually the reverse is found; dilution promptly lowers the Q_{O_2} still further (Fig. 2).

Finally, it is interesting to note the effect of a double addition of lactate (Fig. 4). A suspension in 0.3 per cent lactate at an early stage (1–2 hours) and with a markedly increased respiration is in no way affected by the further addition of one-third its volume of 0.6 per cent lactate. There is no immediate increased oxygen consumption nor any delay in the subsequent fall. If the second portion of lactate is added at the end of nine hours, when the original lactate action has ended and respiration is the same as in a water control, there is some increased consumption but much less than in the control. In one experiment, for example, the Q_{O_2} in both water and lactate suspensions had fallen to 1.5. When new lactate was added to the lactate suspension, this value rose only to 3, while the Q_{O_2} of the water suspension rose to eighteen. If the lactate addition is delayed another 12 hours, however, the water and lactate suspensions being shaken in the usual manner in manometers, the difference is much less marked. Thus, in the same set of runs, lactate added to the water suspension 21 hours after the start again gave a Q_{O_2} of 18, and added to the lactate suspension, one of 12 instead of three. Obviously, a second lactate addition has no effect when added soon after a first one, some effect when added after the initial increase in respiration is past, and close to a maximal effect when added the following day.

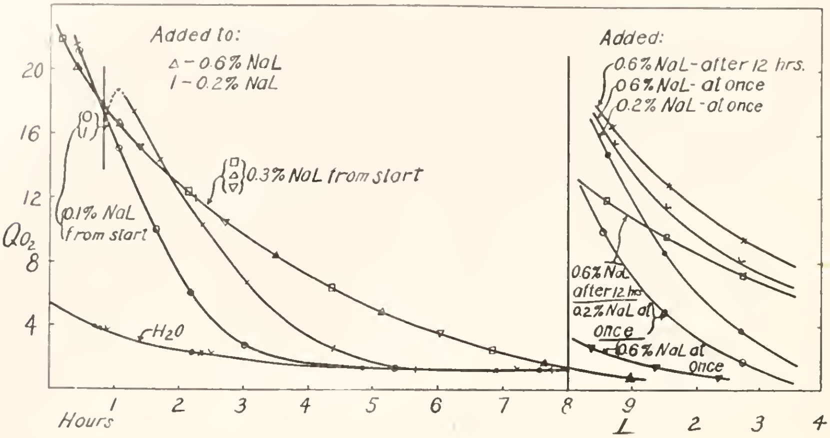

FIG. 4.

When the experiment is carried out with 0.1 per cent lactate and 0.2 per cent lactate added later, the time relations are altered. With these dilutions, a second addition has some effect even two hours after the first. Thus, in one case (Fig. 4), the fall in respiration was delayed an hour or more and some actual increase may have been present at once after the second addition. It is interesting to note further that the actual Q_{O_2} at the moment the addition was made was the same as that of the suspension in 0.3 per cent lactate where a further addition was ineffective. After nine hours, four hours after the respiration of the lactate suspension had become identical with that of the water control, the further addition of 0.2 per cent lactate to each gave the following Q_{O_2} values: water suspension, 19; lactate suspension, fourteen.

These observations strongly suggest that the falling off in rate of oxygen consumption with time is due less to accumulation of some retarding factor than to a temporary exhaustion of some required one. Certainly neither oxygen nor lactate are lacking, and no retarding substance accumulates as the ultimate product. Either some intermediate product is formed rapidly and but slowly further altered, so as to act as a temporary obstacle to the first reaction; or the oxidative mechanisms of the cell are somehow run down by an excessive load and are only gradually restored to the equilibrium state. The effects of sudden dilution are more in accord with the second possibility.

The effect of methylene blue combined with lactate is of interest in this connection. When, in comparable experiments, buffered solutions of lactate alone or with methylene blue are tipped into a water suspension, the maximum Q_{O_2} is consistently greater with the dye absent (Table III). In eight experiments with 0.1 per cent sodium lactate and 0.1 per cent methylene blue the maximum Q_{O_2} averaged 34; in four without the methylene blue, forty. In every run the depressant action of the dye was seen, decreases ranging from 10 to 30 per cent. At two to three hours the curves approached or even crossed for a short time, but at about six hours after the tipping the values were: with methylene blue, $Q_{O_2} = 0.9$, without, $Q_{O_2} = 2.5$.

Methylene blue has, to sum up, a marked early accelerating action on the respiration of *Sarcina* suspensions in water or in glucose solutions. After some hours the rate of oxygen consumption falls to that of control suspensions and is ultimately depressed. With lactate solutions, the depressant action of methylene blue is present at the start, and, in percentage, becomes more marked with the passage of time.

It may be pointed out that for equimolar amounts of glucose and sodium lactate added, the time course of extra oxygen consumption is very different. The glucose leads to a moderate prolonged rise, the lactate to an intense brief one (Fig. 2). This would be in harmony with, though of course not necessitating, a slow split of the glucose molecule to lactic acid or a closely related intermediate followed by a rapid oxidation of this latter. Barron's view (1930) that methylene blue catalyzes primarily the initial splitting of glucose and not the later reactions would then fit the observed results fairly well. The main difficulty with such an interpretation lies in the failure to obtain an accumulation of lactic, pyruvic or other acid stronger than carbonic under anaerobic conditions, as shown in the preceding paper.

Finally, simultaneous addition of glucose and sodium lactate leads to the sharp maximum of lactate followed by the less intense enduring glucose effect. There does not appear to be summation of the two effects—the Q_{O_2} follows the higher single curve (lactate alone or glucose alone) at any time. No separate experiments were performed with optically active lactic acid, the racemate serving in these tests. Since, however, all added lactate can be burned and the Q_{O_2} curve after lactate addition gives no evidence of a discontinuity, it seems probable that both the *d* and *l* forms are easily utilized by this organism.

5. *Thioglycollic Acid.*—A few experiments, made in another connection, have shown that this substance in $0.2M$ concentration doubles the oxygen consumption.

6. *Sodium Cyanide.*—Cyanide, in concentrations up to $N/100$ or a little stronger does not inhibit the oxygen consumption of *Sarcina lutea*. This is true for the usual respiration of suspensions in water, saline, or phosphate buffer, as well as for the increases evoked by methylene blue, lactate, or glucose (Fig. 5). The presence of cyanide appears, in fact, to slightly increase the Q_{O_2} or at least to diminish the rate of fall with time. The average Q_{O_2} of five experiments in water was 1.1 at three hours; in $N/100$ NaCn it was 1.4. The possibility of HCN distilling from the side bulb to the main suspension

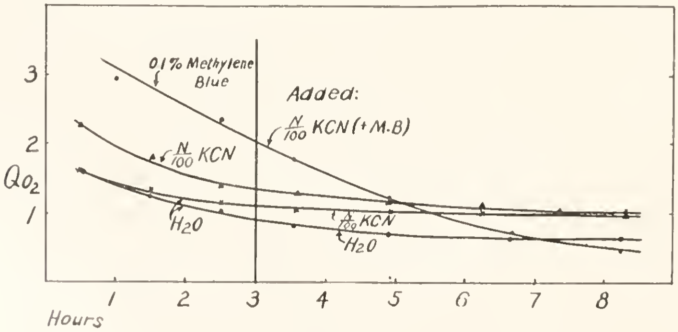

FIG. 5.

prematurely, and so masking a real effect, is excluded. In *N*/30 cyanide, respiration is depressed 1/2 to 2/3, and in *N*/10, 2/3 to 3/4. Rubinstein (1931) has confirmed the absence of cyanide inhibition with water suspensions.[1]

A much less complete absence of cyanide inhibition has been described by Emerson (1927) for the alga *Chlorella*, for in the presence of sugar 10^{-4} *M* cyanide gives 50 per cent inhibition. Lund (1918) has claimed a cyanide insensitivity for *Paramecium*, though this has not passed unchallenged (Hyman, 1919).[2] Pitts (unpublished) has found *Colpidium* to be but slightly sensitive to cyanide. Burnet (1927), studying growth and oxygen consumption of a number of bacterial types under the influence of cyanide, found them divisible into two groups. Most showed a usual degree of sensitivity, but some, as streptococci, were insensitive to cyanide. These groups were also different in their behavior with hydrogen peroxide. Even for vertebrate tissues a complete inhibition of respiration by cyanide is not the rule. *N*/100 does not inhibit the respiration of frog nerve by more than 80 per cent (Gerard, 1930), and Dixon and Elliot (1929) obtained similar results with a variety of mammalian tissues, though Alt (1930) and Warburg (1930) sharply criticize their work. The objections raised certainly do not apply to the present results. *Sarcina lutea* apparently carried on a fairly typical aerobic metabolism, using oxygen freely to burn the usual organic molecules. The almost complete insensitivity of its respiratory catalytic system to cyanide would seem to preclude a too broad generalization as to the ferroactive nature of oxidative enzymes.[3] The action of carbon monoxide is likewise atypical.

7. *Carbon Monoxide.*—Carbon monoxide containing 5 per cent

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[1] Working with descendants of the original strain, over a year later, Barron obtained definite inhibition of respiration by cyanide. I repeated my original experiments, using the identical techniques previously employed, and also found inhibition was now present. In water suspensions *N*/100 cyanide gave depressions of 50 per cent or more, in glucose even greater ones. The depressions were, however, of short duration and respiration returned to normal in an hour and sometimes became excessive later. Presumably the organisms had altered in the interim due, perhaps, to unknown differences in the culture conditions.

[2] This has now been confirmed. Gerard and Hyman, *Am. Jour. Physiol.*, in press.

[3] It may be mentioned here that Dr. E. S. G. Barron and I have demonstrated by means of the spectroscope that cytochrome is present in these cells. Further, it is changed easily between the oxidized and reduced states by bubbling in oxygen or nitrogen. In the presence of *N*/100 KCN, however, the pigment remains reduced even in a stream of oxygen. This appears as evidence that cytochrome is not an essential link in the chain of respiratory substances of this cell, since respiration proceeds as normally after its oxidation is blocked. We are further investigating the oxidizing mechanisms of *Sarcina*.

oxygen was used in all experiments and the thermostat covered to exclude daylight. A 200-watt tungsten bulb, immersed in the white-walled thermostat, within 2 to 10 inches of the manometer chambers, was turned on at intervals of 20 minutes to 1 hour.

Water suspensions of *Sarcina* respired alike in air and in the monoxide mixture. Light had no obvious effect on either. When glucose (1.0 per cent) or lactate (1.0 per cent) was added to such water suspensions, an inhibiting action of the CO appeared. In the case of glucose this was slight, the increased oxygen consumption in CO being over three-fourths that in air. With lactate, the inhibition was marked, the increase in monoxide being less than one-fifth that in air, but inadequate oxygen may have contributed to the effect. In both cases, glucose or lactate added, when the carbon monoxide mixture was later replaced by air, the full rate of oxygen consumption promptly appeared. The presence of light, of the intensity used, had no reversing action on the inhibited lactate oxidation. There was a suggestion of such an effect in increasing the respiration of a suspension in glucose in the presence of CO, but an entirely similar augmentation appeared also in air (Fig. 6). Rubenstein (1931) has found that light increases the respiration of *Sarcina lutea* at this temperature.

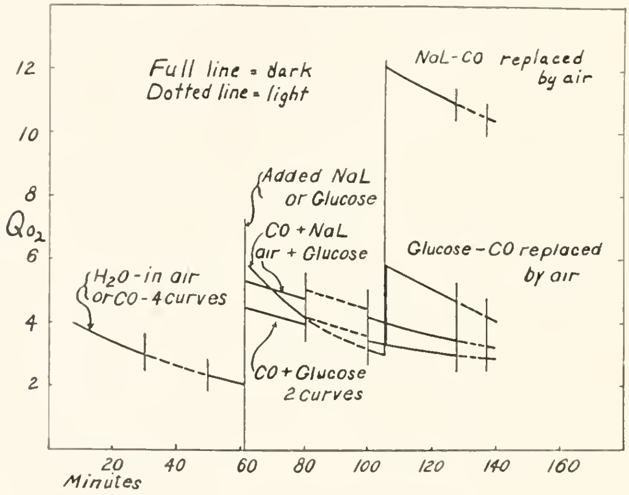

FIG. 6.

DISCUSSION

These results raise several interesting questions regarding the chemical dynamics involved. Q_{O_2} is a measure of reaction velocity, yet this is independent of the concentration of the primary reactants over a wide range. Thus 0.05 and 1.1 per cent lactate give the same maximal rates, but both rates decline following the maximum. For the lower concentrations, at least, the rate must fall with decreasing amount of substrate. Probably at sufficiently low initial concentrations, the maximum reached would vary with concentration, until an asymptotic value had been reached, beyond which concentration has no effect. The critical concentration of lactate cannot be over 0.05 per cent, but may be very close to this. The decline in rate after the maximum, when considerably larger amounts of lactate are present, cannot, as in the previous case, be due to diminution in concentration of the lactate, for this must remain for long periods above the critical value. Evidence has also been presented to show that accumulation of end products is not the important factor leading to slowing, though temporary piling up of intermediates might play a rôle. Since, in the simplified reaction:

$$\text{Substrate} + O_2(+ \text{Respiratory Catalyst}) \rightarrow CO_2 + H_2O + \text{End Products},$$

the velocity appears to be independent of substrate, oxygen, or end product concentration, its fall might be attributed to interference with the respiration-catalyzing system. This might mean destruction or out-diffusion (suggested by the fall in Q_{O_2} on dilution) from the cell of one of the key substances in the respiratory chain more rapidly than it is replaced.

The situation with respect to glucose is analogous though quantitatively different; and the ultimate depressant action of methylene blue can also be laid at the door of an injured catalytic system.

Meyerhof (1912) has shown that the oxygen consumption of acetone-yeast is doubled by the addition of methylene blue, whereas that of the intact cells is depressed by it; and similar results were obtained with staphylococci (1917). Barron (1930) likewise has found little or no stimulating action of methylene blue on normal tissues, though it does increase the respiration of cancer and other aerobically glycolysing material. Gerard (1930) has found that medullated nerve, on the contrary, though lacking aerobic glycolysis, is stimulated by methylene blue to a marked respiratory increase, and Chang (unpublished) has confirmed this for non-medullated nerve and with cresyl blue. *Sarcina* in this respect resembles nerve. It may be noted in passing that methylene blue largely or entirely reverses the cyanide inhibition of respiration in the case of staphylococci (Meyerhof) and nerve (Gerard, Chang). There is no evidence that the yellow pigment of *Sarcina* acts in a similar way, though such activity might contribute to the cyanide insensitivity of this organism.

SUMMARY

The Q_{O_2} of washed *Sarcina lutea* in water suspension at 22° C. in the relatively steady state averages 2.5. There is no untoward effect of crowding in heavy suspensions. The presence of NaCl does not modify the Q_{O_2} until $M/5$ concentrations are reached, when a slight depression may result. The Q_{O_2} is also unaffected by pH, at least between 7 and 8, or by the presence of phosphate buffer mixtures.

Glucose addition causes a marked increase in the Q_{O_2}, the maximum being largely independent of glucose concentration. The extra oxygen used under the influence of glucose may be more than that required to fully oxidize it which, with other evidence, suggests a "specific dynamic action."

Sodium lactate may increase the Q_{O_2} in water suspension over twenty-fold. Its addition is always followed by a great rise of the respiratory rate, the maximum reached being independent of lactate concentration, at least between 0.05 and 2.0 per cent. The respiration falls rapidly back to normal after addition of small amounts, and the extra oxygen consumed accounts for full oxidation of the added lactate, both *d* and *l* forms. With larger concentrations of lactate, the increased respiration also falls after the initial maximum, but more slowly. This fall is not primarily due to removal of lactate nor accumulation of end products.

Methylene blue added to a suspension in water doubles respiration at first, later depresses it. Added to one in glucose solution the same sequence appears. The Q_{O_2} in glucose is increased only 50 per cent by the dye, but this is an absolute increase over twice that in water; so that glucose and methylene blue added together to a water suspension cause a greater increase in respiration than the sum of their separate effects. The dye added to lactate solutions seems to be depressant from the start.

Thioglycollic acid doubles the respiration of a buffered water suspension.

Sodium cyanide causes no inhibition of respiration up to concentrations of $M/100$ or somewhat stronger. This is true for the low respiration of suspensions in water, saline, or phosphate buffer and for the increases evoked by methylene blue, lactate and glucose. Extremely strong cyanide concentrations do depress, but even $M/10$ NaCN does not abolish more than 2/3 to 3/4 of the total respiration.

Carbon monoxide containing 5 per cent oxygen has no effect on the respiration of a water suspension, but somewhat inhibits the

increase in glucose and largely that in lactate. Light has little if any effect on the inhibition.

The metabolism of *Sarcina lutea* is compared with that of other cell types.

My thanks are due Miss Ruth Bilger for valuable technical assistance.

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THE OXYGEN TENSION-OXYGEN CONSUMPTION CURVE OF UNFERTILIZED ARBACIA EGGS

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Gerard (1931) has developed equations relating the external oxygen pressure to the oxygen consumption, cell diameter and diffusion constants for individual cells. This work was undertaken to obtain experimental data under favorable conditions for comparison with the theoretical derivation. For this the sea urchin egg is beautifully adapted, as it is homogeneously spherical, regular, and its resting rate of oxygen consumption can be suddenly increased by fertilization. Its effective diameter can also be varied by allowing a greater or lesser amount of the egg jelly to remain about each cell. It is our intention to investigate the effect of varying these several factors, but for the present the results with simple, clean, unfertilized eggs are to be presented.

METHODS

Oxygen consumption was determined by the usual Warburg technique, using small conical vessels of about three cc. capacity, containing an inset for alkali. The experiments were conducted at 24.7° C. and the shaking of the manometers was demonstrated to be adequate for diffusion equilibrium even at the low oxygen pressures used. Gas mixtures were prepared by mixing nitrogen and air over water and, for the smaller concentrations, by direct mixing in the manometer as described by Gerard and Falk (1931). When the mixing was done over water, the composition was checked by analysis in the Haldane apparatus. Passing the gas through the manometers with gentle shaking for fifteen minutes sufficed to bring the fluid into complete equilibrium with it.

The *Arbacia* eggs were obtained by picking out the gonads of about twenty animals, allowing them to shed into sea water, and straining through cheese cloth. The eggs were allowed to settle and the supernatant water decanted, giving a heavy suspension. They were not further washed, but controls showed no difference in respiration between washed and unwashed suspensions, and fertilization tests yielded over 90 per cent cleavage. The concentration of eggs in the suspension was determined by means of a hemocytometer, and half a cc. was used for each manometer. The egg diameters, as determined by an ocular micrometer, averaged 77 micra, variation 72 to 80.

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[1] It is a pleasure to acknowledge the guidance, help and suggestions of Professors R. S. Lillie and R. W. Gerard, under whose supervision this work was done. To Dr. Walter S. Root my thanks are due for many of the gas analyses.

Significant readings were made for three and a half to four hours and the viability of the eggs again checked by fertilization. In each experiment parallel runs were made of the respiration in air or oxygen and at the lower oxygen pressures, so that the latter values could be expressed as a direct percentage of the former.

TABLE I

Oxygen Tension mm. of Hg	Oxygen Consumption per cent in air	No. of Experiments
2.0	43.0	13
6.1	54	7
9.9	69	2
20.5	92	3
42.0	92	5
86.0	101	3
160 (air)	100 ($Q_{O_2} = 33.6$)	24
760	100	3

RESULTS

The average Q_{O_2}, expressed as cubic millimeters of oxygen consumed per million eggs per hour, was 33.6 in air (extremes 17 to 51; 24 experiments), the lower values being obtained toward the end of the breeding season. At oxygen tensions of 70–80 mm. or higher, up to pure oxygen, the consumption was the same as in air. Below 70–80 mm. the consumption fell off with pressure, as indicated in Table I. Beginning at the oxygen tension of 20 mm., the oxygen consumption fell very rapidly with a decrease in oxygen tension. Surprisingly, the same type of curve, with the turning point coming at the same oxygen tension has been obtained by Amberson (1928), on *fertilized* eggs by a different method.

The oxygen consumption was found to proceed at a constant rate up to about four and one-half hours, at which time there was a sudden increase in oxygen consumption, both in air and in lower concentrations of oxygen. This seems to be related to a fairly abrupt cytolysis of large numbers of the cells. A few experiments were carried out with fertilized sea urchin eggs to determine the possible existence of a rhythm of oxygen consumption analogous to the rhythm of production of CO_2 reported by Lyon (1904). The experiments showed the fertilized eggs to have a respiration five times that of the unfertilized ones, confirming Warburg (1908) and Loeb and Wasteneys (1910); but, within the limits of accuracy of the method, the rate of respiration was constant through the first, second and third cleavage periods.

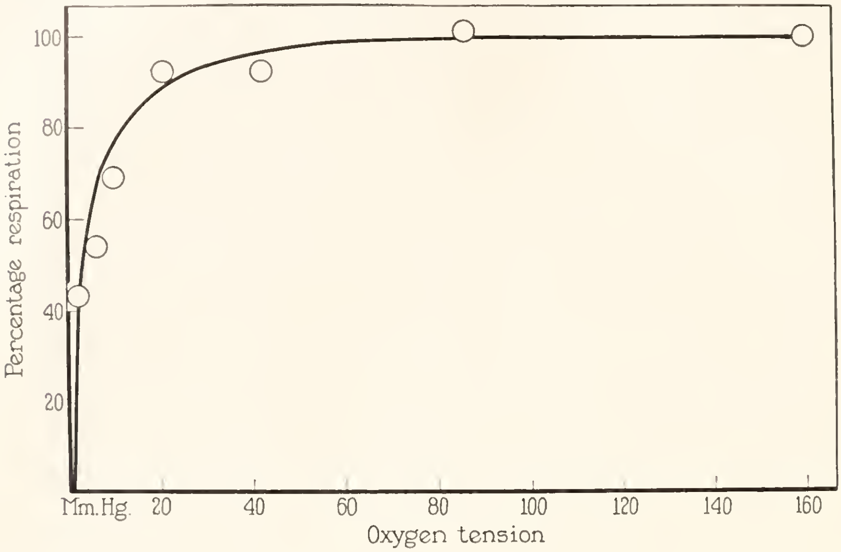

FIG. 1.

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OXYGEN DIFFUSION INTO CELLS

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Krogh (1919) determined the coefficients of diffusion of oxygen through surviving frog muscle and through fascia by using these as tissue membranes and measuring the gas diffusing through under given conditions. He applied the measured values in a formula relating capillary size and number to the oxygen needs of muscle *in vivo*.

These constants have been utilized by subsequent workers for substitution in other equations applying to the equilibrium state. Warburg (1923) developed an equation relating the necessary oxygen pressure to the metabolism and thickness of a slice of isolated tissue. Fenn (1927) and Gerard (1927) independently presented analogous equations for a cylinder of tissue, and A. V. Hill (1928) has extensively surveyed the general question of diffusion in tissues and developed several formulæ for the attaining of equilibrium as well as for the equilibrium state. Applying these equations to the specific question of diffusion of phosphate ion (Stella, 1928) and lactate ion (Eggleton, Eggleton and Hill, 1928), the values for the diffusion constants obtained suggested that these ions moved mainly in the intercellular fluid and with much greater difficulty across cell boundaries.

Should a similar situation obtain for oxygen, the calculations based on Krogh's constant might be considerably in error, and in any case, since this value has been so widely used, any further checks on its magnitude should be of value.

The simplest situation for studying oxygen penetration into living cells is offered, of course, by the unicellular organisms, which present, mainly, the problem of the sphere. It was the original intent of this work to evaluate the diffusion constant for cells as the only unknown in a simple equation, but it soon became apparent that a more searching examination of the assumptions made in such a simplified derivation was necessary. The elementary equation for diffusion into a sphere is easily derived as follows:

At equilibrium, the amount of oxygen diffusing into a mass of tissue must equal the amount consumed by it in the same time. For unit time, the inward diffusion depends on the surface area ($4\pi r^2$ for the sphere); the diffusion coefficient, D; and the gradient of oxygen concentration along a radius, dC/dr, C being the oxygen concentration at radius r. The consumption of oxygen by a sphere of indeterminate radius, r, is the product of the measured consumption per unit volume $\left(= \frac{\text{mass}}{\text{sp. gr.}} = \text{mass} \times 0.95 \text{ for most protoplasm}\right)$, A, times the volume, $\frac{4}{3}\pi r^3$. At equilibrium, then

$$4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi r^3 A$$

or

$$\frac{dC}{dr} = \frac{Ar}{3D}; \tag{1}$$

which gives on integration

$$C = \frac{Ar^2}{6D} + K.$$

At $r = 0$, $C = K$ and K therefore represents the oxygen concentration at the center of the sphere.

In the case of most tissues so far studied, the oxygen consumption, A, has been found to be independent of the oxygen pressure over a wide range. The assumption has been made in this derivation that consumption in any one region is independent of oxygen concentration, provided this is greater than zero. Then for the critical condition where the oxygen concentration just reaches zero at the center of the sphere, $K = 0$, and the oxygen concentration at any level in the sphere is

$$C = \frac{Ar^2}{6D};$$

and at the surface, $r = r_0$, the critical concentration of oxygen needed to just insure a supply at the center is

$$C_0 = \frac{Ar_0^2}{6D} \tag{2}$$

and

$$D = \frac{Ar_0^2}{6C_0}. \tag{3}$$

A may be fairly simply determined with one of the available methods of measuring oxygen consumption, and C_0 is that concentration of oxygen in the medium which just gives full values of oxygen consumption. With lower concentrations the observed oxygen consumption begins to fall off. If the radius of the cell is known, a simple means is available for obtaining the oxygen diffusion coefficient, or for bringing out the invalidity of assumptions as to the conditions of respiration.

It may be stated at once that the results of applying this simple equation to a variety of cells, as compared with results experimentally obtained, show such great variation as to indicate that other factors than the simple diffusion constants must be involved. It is necessary, then, to examine in some detail the assumptions made in the above derivation. Probably the most important is that just expressed, namely that the oxygen consumption of any region is independent of the concentration of oxygen at that region. This will be examined later. Other assumptions of little probable importance in the case of tissues may become extremely significant in the case of the individual cell. These are, first, that the oxygen consumption per individual region is a constant throughout the cell, that is, that the total consumption divided by the total volume gives the true consumption of each region. This is certainly not necessarily true, as the rate of respiration of a nuclear region or a cortical region might clearly be very much greater than that in other regions. It is also implicitly assumed that the diffusion constant, whether equal to that of Krogh or not, is at least constant throughout the cell. This also is not at all certain; in fact, in view of the previous discussion, it is entirely possible that the constant would vary widely across the membrane and in the cell interior.[1]

The mathematical development which follows is designed first to include all possible cases in which diffusion or consumption in two concentric regions of the cell are not assumed to be equal; and, following that, the more difficult case of the dependence of consumption on oxygen concentration will be considered. In this treatment I have called freely upon the expert assistance, generously given, of Dr. Walter Bartky of the Department of Astronomy at this University. The final section of the paper will apply the equations derived to the data available in the literature as well as to our own measurements, in an attempt to evaluate the conditions actually existing as regards respiration. Obviously, the derivations apply equally to other diffusable substances formed in and leaving or entering and utilized by the cell.

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[1] The further complication, in many cells, of protoplasmic streaming cannot be handled rigorously. It is probably not significant in bacteria, yeast, or even *Arbacia* eggs; and when it is present, the stirring action would act as a decrease in radius in lowering the diffusion limit.

1. *Oxygen Consumption not Alike in All Portions of A Sphere*

a. Limited to a central core (nuclear respiration).—Let Q = the total oxygen consumed by the cell; C = the oxygen concentration at radius, r; r_0 = radius at cell surface; r' = radius of respiring region.

The oxygen diffusing into a sphere centered at the cell center and of any radius, r, equals $4\pi r^2 D \frac{dC}{dr}$. When $r \geqq r'$, all oxygen consumed by the cell must pass through to the region of consumption, so:

$$4\pi r^2 D \frac{dC}{dr} = Q.$$

The consumption per unit volume in the non-respiring portion of the cell is zero; of the respiring portion: $\frac{Q}{\frac{4}{3}\pi r'^3}$.

When $r \leqq r'$, then, the oxygen passing the shell must equal the volume within this shell times the consumption per unit volume, or

$$4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi r^3 \frac{Q}{\frac{4}{3}\pi r'^3}.$$

Then

$$\frac{dC}{dr} = \frac{Q}{4\pi r^2 D}, \qquad \text{when} \quad r \geqq r', \tag{4a}$$

$$\frac{dC}{dr} = \frac{rQ}{r_0^3\, 4\pi D}, \qquad \text{when} \quad r \leqq r'. \tag{4b}$$

Integrating:

$$C = -\frac{Q}{4\pi Dr} + K_1, \qquad r \geqq r', \tag{5a}$$

$$C = \frac{Qr^2}{8\pi D r_0^3} + K_2, \qquad r \leqq r'. \tag{5b}$$

For the critical condition, $C = 0$ when $r = 0$ and $C > 0$ for $r > 0$, then $K_2 = 0$; and since C is continuous at $r = r'$,

$$-\frac{Q}{4\pi Dr'} + K_1 = \frac{Qr'^2}{8\pi Dr'^3}$$

or

$$K_1 = \frac{3Q}{8\pi Dr'}.$$

So that, from (5*a*),

$$C = \frac{Q}{4\pi D}\left(\frac{3}{2r'} - \frac{1}{r}\right), \qquad r \geqq r'.$$

At the cell surface $r = r_0 \geqq r'$,

$$C_c = \frac{Q}{4\pi D}\left(\frac{3}{2r'} - \frac{1}{r_0}\right). \tag{6}$$

(C_c = oxygen concentration needed at the surface, radius = r_0, to just insure full penetration, in this case when all the oxygen is consumed within a sphere of radius r'.)

For the special case when $r' = r_0$, or oxygen consumption is uniform throughout the cell, this reduces to

$$C_0 = \frac{Q}{8\pi D r_0}$$

or, since A = consumption per unit volume,

$$A = \frac{Q}{\frac{4}{3}\pi r_0^3},$$

and therefore

$$C_0 = \frac{A r_0^2}{6D},$$

the original equation.

In general,

$$\frac{C_c}{C_0} = \frac{3\,r_0}{r'} - 2. \tag{7}$$

It is readily seen from equation (7) that as r' decreases relative to r_0, the critical oxygen pressure needed, C_c, increases rapidly relative to C_0. When $r' = \frac{1}{2}r_0$, $C_c = 4C_0$; when $r' = \frac{1}{4}r_0$, $C_c = 10C_0$.

b. Oxygen consumption limited to an outer shell (cortical region).—By an entirely analogous derivation it can be shown for this case that

$$\frac{C_c}{C_0} = \frac{(r_0 + 2r')(r_0 - r')}{r_0^2 + r_0 r' + r'^2}. \tag{8}$$

From this equation it appears that as the respiring shell of thickness $(r_0 - r')$ thins, $\frac{C_c}{C_0}$ decreases towards zero. When

$$r' = 0, \frac{C_c}{C_0} = 1; \quad r' = \tfrac{1}{2}r_0, \frac{C_c}{C_0} = 0.57; \quad r' = \tfrac{3}{4}r_0, \frac{C_c}{C_0} = 0.27;$$

$$r' = 0.9r_0. \qquad \frac{C_c}{C_0} = 0.10.$$

c. Oxygen consumption present throughout cell, but more or less intense in an outer shell.—This will be considered in the general case below. See Equations 17 and 18.

2. *Diffusion Constant Different in Central and Cortical Parts of Cell*

If the diffusion constant is smaller at one region than another—as that of a relatively impermeable membrane is small compared to that of a permeable cell interior—the following derivation becomes necessary.

Let the diffusion coefficient be D' for the cell interior from $r = 0$ to $r = r'$; and D for a superficial shell, $r = r'$ to $r = r_0$. Then, by a familiar derivation,

$$\text{When} \quad r \leqq r', \qquad 4\pi r^2 D' \frac{dC}{dr} = \frac{4}{3}\pi r^3 A\,; \qquad \frac{dC}{dr} = \frac{Ar}{3D'}$$

$$\text{When} \quad r \geqq r', \qquad 4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi r^3 A\,; \qquad \frac{dC}{dr} = \frac{Ar}{3D}\,.$$

Integrating between limits, and assuming $C = 0$ for $r = 0$, and $C > 0$ for $r > 0$,

$$C' = \frac{Ar'^2}{6D'},$$

$$C_0 - C' = \frac{A(r_0^2 - r'^2)}{6D},$$

where C' is the concentration at r'. Combining,

$$C_0 = \frac{A}{6}\left(\frac{r_0^2 - r'^2}{D} + \frac{r'^2}{D'}\right), \tag{9}$$

which reduces to the simple equation (2) when $D = D'$ or $r' = r_0$.

It is obvious from (8) that if the cell membrane is thin compared to the cell radius, that is, r' is nearly as large as r_0, its diffusion constant, D, must be very small in relation to that of the interior, D', to affect C_0. For example, to double C_0 when $r' = 0.9r_0$, D must be $0.16D'$; and when r' is $0.99r_0$, $D = 0.02D'$. To increase C_0 ten times: for $r' = 0.9r_0$, $D = 0.012D'$; for $r' = 0.99r_0$, $D = 0.002D'$. Since it appears from the work of the Eggletons and Hill (1928) that the diffusion coefficient for ions across the cell surface may be one hundredth as large as in the cell or intercellular fluid, this factor obviously might be of importance for oxygen diffusion. But unionized molecules appear to penetrate membranes much more easily than do ions, so that the analogy may not hold.

In all the above derivations the critical oxygen pressure at the cell surface has been calculated. That is, assuming that the oxygen consumption of the whole cell remains at its maximum value, what is the necessary external pressure as the various conditions within the cell are changed? It is next desirable to investigate the oxygen consumption-external oxygen pressure curve below this critical value and to examine its shape for the simplest situation.

Let r_1 represent the depth of oxygen penetration, *i.e.*, $C = 0$ for values of r such that $r_1 \geqq r \geqq 0$, and $C > 0$ for $r > r_1$. And let C_s represent the oxygen concentration at the surface, $r = r_0$.

Then, as the respiring volume $= \frac{4}{3}\pi(r^3 - r_1^3)$,

$$4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi A(r^3 - r_1^3),$$

$$\frac{dC}{dr} = \frac{A(r^3 - r_1^3)}{3Dr^2},$$

$$C_s = \frac{A}{3D}\int_{r_1}^{r_0} \frac{r^3 - r_1^3}{r_2},$$

$$= \frac{A}{3D}\left(\frac{r_0^2}{2} + \frac{r_1^3}{r_0} - \frac{3}{2}r_1^2\right). \tag{10}$$

Similarly, the oxygen consumed is:

$$Q = 4\pi\int_{r_1}^{r_0} Ar^2 dr,$$

$$Q = \frac{4\pi A}{3}(r_0^3 - r_1^3). \tag{11}$$

Take units such that $r_0 = 1$, and that $C_s = C_0 = 1$ for $r_1 = 0$, and also that $Q = 1 = Q_0$ for $r_1 = 0$. Note that with this selection of units, $\frac{A}{D} = 6$, $A = \frac{3}{4\pi}$, $D = \frac{1}{8\pi}$. The relation between Q and C for the general case is then expressed in terms of r as a parameter: From (10)

$$C_s = 1 - r_1^2(3 - 2r_1),$$

and from (11)

$$Q = 1 - r_1^3,$$

which give the following values (Table I). The corresponding curve is given in Fig. 1.

Still more generally, assuming only that A is constant as regards C, the following derivation includes all cases involving a single concentric zoning of the cell as regards respiration and diffusion.

Let r' represent the surface separating two cell zones.

Let D' and A' represent the diffusion coefficient and unit oxygen consumption for the nuclear region, $r < r'$; and similarly D and A represent these values for the cortical region, $r > r'$.

TABLE I

$\frac{C_s}{C_0}$	$\frac{r_1}{r_0}$	$\frac{Q}{Q_0}$
1.00	0.0	1.00
0.90	0.2	0.99
0.65	0.4	0.94
0.50	0.5	0.87
0.35	0.6	0.78
0.22	0.7	0.66
0.10	0.8	0.49
0.04	0.9	0.27
0.00	1.0	0.00

Let r_1 represent, as previously, the depth of oxygen penetration. In the following it is assumed that $r_1 \leqq r'$, for when $r_1 > r'$ the situation reduces to the simple cases previously considered, inasmuch as no oxygen reaches the inner sphere where conditions are different.

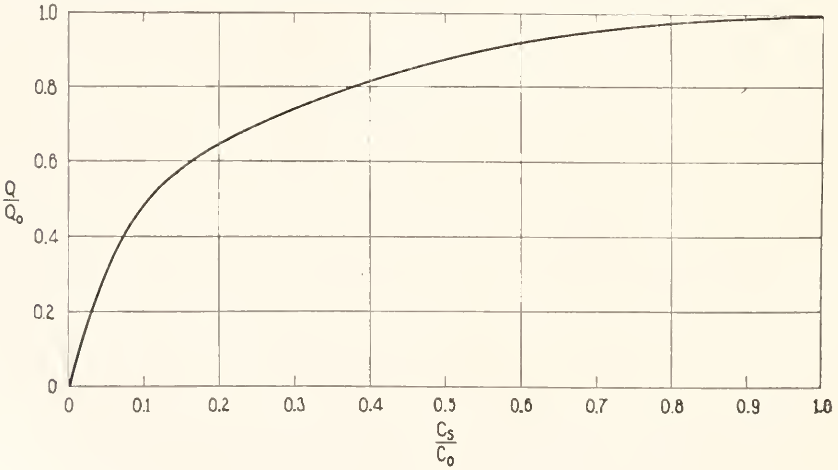


FIG. 1.

Let C' represent the oxygen concentration at $r = r'$; C_s the oxygen concentration at the cell surface, $r = r_0$; and C_c the critical surface concentration to just insure complete penetration. For the simple case, $C_c = C_0$.

Then the derivation, along the previous lines, is:

$$\text{For } r < r', \qquad 4\pi r^2 D' \frac{dC}{dr} = \frac{4}{3}\pi A'(r^3 - r_1^3); \tag{12a}$$

$$r > r', \qquad 4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi(r'^3 - r_1^3)A' + \frac{4}{3}\pi A(r^3 - r'^3). \tag{12b}$$

Simplifying:

$$\text{For } r < r', \qquad \frac{dC}{dr} = \frac{A'}{3D'}\,\frac{(r^3 - r_1^3)}{r^2},$$

and integrating from r_1 to r':

$$C' = \frac{A'}{3D'}\left(\frac{r'^2}{2} + \frac{r_1^3}{r'} - \frac{3r_1^2}{2}\right). \tag{13}$$

Similarly:

$$\text{For } r > r', \qquad \frac{dC}{dr} = \frac{A'(r'^3 - r_1^3)}{3Dr^2} + \frac{A(r^3 - r'^3)}{3Dr^2}$$

and integrating from r' to r_0:

$$C_s - C' = \frac{A'(r'^3 - r_1^3)}{3D}\left(\frac{1}{r'} - \frac{1}{r_0}\right) + \frac{A}{3D}\left(\frac{r_0^2}{2} - \frac{r'^3}{r_0} - \frac{3}{2}r'^2\right). \tag{14}$$

From (13) and (14)

$$C_s = \frac{A'(r'^3 - r_1^3)}{3D}\left(\frac{1}{r'} - \frac{1}{r_0}\right) + \frac{A}{3D}\left(\frac{r_0^2}{2} + \frac{r'^3}{r_0} - \frac{3}{2}r'^2\right) + \frac{A'}{3D'}\left(\frac{r'^2}{2} + \frac{r_1^3}{r'} - \frac{3}{2}r_1^2\right). \tag{15}$$

Equation (15) may be explored as follows:
For $r_1 = 0$, that is, at the critical external oxygen pressure,

$$C_c = \frac{A'r'^3}{3D}\left(\frac{1}{r'} - \frac{1}{r_0}\right) + \frac{A}{3D}\left(\frac{r_0^2}{2} + \frac{r'^3}{r_0} - \frac{3}{2}r'^2\right) + \frac{A'r'^2}{6D'}. \tag{16}$$

For $r_1 = r' = 0$

$$C_c = \frac{Ar_0^2}{6D}.$$

For $r_1 = 0$, $r' = r_0$

$$C_0 = \frac{A'r_0^2}{6D'}.$$

For $r_1 = r'$

$$C_s = \frac{A}{6D}\left(r_0^2 + \frac{2r'^2}{r_0} - 3r'^2\right). \quad \text{(See Equation 10.)}$$

For $r_1 = r' = r_0$

$$C_s = 0.$$

For $D = D'$

$$\begin{aligned} C_s &= \frac{A'}{3D}\left[\frac{3}{2}(r'^2 - r_1^2) - \frac{r'^3 - r_1^3}{r_0}\right] + \frac{A}{3D}\left(\frac{r_0^2}{2} + \frac{r'^3}{r_0} - \frac{3}{2}r'^2\right) \\ &= \frac{(A - A')r'^2}{6D}\left(\frac{2r'}{r_0} - 3\right) + \frac{Ar_0^2}{6D} + \frac{A'r_1^2}{6D}\left(\frac{2r_1}{r_0} - 3\right). \end{aligned} \tag{17}$$

If $A = A'$, or $r_1 = r'$,

For $r_1 = 0$, $$C_s = \frac{A}{6D}\left(r_0^2 + \frac{2r_1^3}{r_0} - 3r_1^2\right). \qquad \text{(See Equation 10.)}$$

$$C_c = \left(\frac{A - A'}{6D}\right) r'^2 \left(\frac{2r'}{r_0} - 3\right) + \frac{Ar_0^2}{6D}. \tag{18}$$

If $A = A'$,

$$C_0 = \frac{Ar_0^2}{6D}.$$

For $A = A'$

$$C_s = \frac{A}{3D}\left[\frac{r_0^2 - r'^2}{2} - r_1^3\left(\frac{1}{r'} - \frac{1}{r_0}\right)\right] + \frac{A}{3D'}\left(\frac{r'^2}{2} + \frac{r_1^3}{r'} - \frac{3}{2}r_1^2\right)$$

$$= \frac{A}{6}\left(\frac{1}{D'} - \frac{1}{D}\right)\left(r'^2 + \frac{2r_1^3}{r'}\right) + \frac{A}{6}\left(\frac{r_0^2 - \frac{2r_1^3}{r_0}}{D} - \frac{3r_1^2}{D'}\right). \tag{19}$$

If $D = D'$ or $r_1 = r'$,

For $r_1 = 0$, $$C_s = \frac{A}{6D}\left(r_0^2 - \frac{2r_1^3}{r_0} - 3r_1^2\right) \qquad \text{(See Equation 10.)}$$

$$C_c = \frac{A}{3D}\left(\frac{r_0^2 - r'^2}{2}\right) + \frac{Ar'^2}{6D'}$$

$$= \frac{A}{6}\left(\frac{r_0^2 - r'^2}{D} + \frac{r'^2}{D'}\right). \qquad \text{(See Equation 9.)}$$

If $D = D'$,

$$C_0 = \frac{Ar_0^2}{6D}.$$

The expression for oxygen consumed per cell is:

$$Q = 4\pi A' \int_{r_1}^{r'} r^2 dr + 4\pi A \int_{r'}^{r_0} r^2 dr$$

$$= \frac{4}{3}\pi[A'(r'^3 - r_1^3) + A(r_0^3 - r'^3)]. \tag{20}$$

Table II and Fig. 2 give the $Q - C_s$ curves obtained for various relationships of $\frac{r'}{r_0}, \frac{D}{D'}, \frac{A}{A'}$.

Consider finally the general case where $A \alpha F(C)$ and is not always a constant, though approximately so for high values of C_0. The amount of oxygen crossing a spherical surface of radius r is $4\pi r^2 D \frac{dC}{dr}$;

therefore that consumed in a shell of internal radius $= r$, external radius $= r + dr$, is

$$4\pi\left(r^2 D\frac{dC}{dr}\right)\Big|_{r+dr} - 4\pi\left(r^2 D\frac{dC}{dr}\right)\Big|_{r}.$$

This is equal to the consumption per unit volume times the volume: $A 4\pi r^2 dr$.

A is some function of $C = DF(C)$, where $F(C)$ does not decrease with increase in C, *i.e.*, a monotonically increasing function of C. Then

$$\left(r^2 D\frac{dC}{dr}\right)\Big|_{r+dr} - \left(r^2 D\frac{dC}{dr}\right)\Big|_{r} = DF(C)r^2dr$$

or

$$\frac{d\left(r^2 D\frac{dC}{dr}\right)}{dr} = r^2DF(C);$$

and, assuming D independent of C and r,

$$\frac{d\left(r^2 \frac{dC}{dr}\right)}{dr} = r^2F(C). \tag{21}$$

The total oxygen consumption of a cell is

$$Q = \int_0^{r_0} A 4\pi r^2 dr$$
$$= 4\pi D \int_0^{r_0} F(C)r^2dr. \tag{22}$$

Q as a function of C_0 is an experimentally observable relationship, from which it is desired to obtain $F(C)$. Consider the most general case, that is, either $C = 0$ for $r = r_1$, where $r_1 \geqq 0$, or $C > 0$ for all values of r. For this latter, always take $r_1 = 0$ in what follows. Integrating (21) between r_1 and r,

$$r^2\frac{dC}{dr} = \int_{r_1}^{r} r^2F(C)dr,$$

$$\frac{dC}{dr} = \frac{1}{r^2}\int_{r_1}^{r} r^2F(C)dr;$$

and integrating between r and r_0,

$$C_0 - C = \int_r^{r_0}\frac{1}{r^2}\left(\int_{r_1}^{r} r^2F(C)dr\right)dr,$$

or

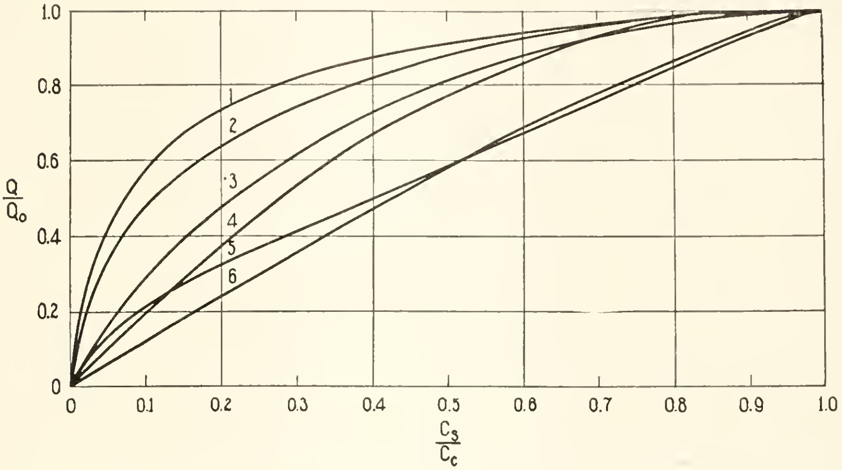
$$C = C_0 - \int_r^{r_0}\frac{1}{r^2}\left(\int_{r_1}^{r} r^2F(C)dr\right)dr. \tag{23}$$

TABLE II

	For $A = A'$					$A = 5A'$					
$\frac{Q}{Q_0}$	C_s/C_c										
	$r' = 0.9\, r_0$ $D = 0.01\, D'$	$r' = 0.98\, r_0$ $D = 0.1\, D'$	$r' = 0.67\, r_0$ $D = 0.01\, D'$	$D = D'$	$r' = 0.98\, r_0$ $D = 0.01\, D'$	$r' = 0.67\, r_0$ $D = D'$		$r' = 0.98\, r_0$ $D = 0.1\, D'$		$r' = 0.9\, r_0$ $D = 0.01\, D'$	
						$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(1.00)	(19.8)	(1.37)	(56.1)	(1.00)	(5.03)	(3.83)	(2.04)	(1.24)	(1.46)	(2.08)	(3.10)
0.99	0.97	0.945	0.98	0.90	0.96	1.00	0.95	1.00	0.92	1.00	0.99
0.94	0.91	0.72	0.88	0.65	0.86	0.99	0.83	0.95	0.70	0.97	0.95
0.875	0.85	0.60	0.77	0.50	0.80	0.98	0.75	0.90	0.62	0.94	0.90
0.79	0.73	0.47	0.61	0.35	0.69	0.945	0.68	0.83	0.50	0.90	0.83
0.66	0.58	0.33	0.38	0.22	0.56	0.80	0.27	0.72	0.37	0.84	0.73
0.49	0.39	0.21	0.18	0.10	0.40	0.64	0.13	0.59	0.25	0.76	0.61
0.27	0.15	0.09	0.05	0.03	0.21	0.35	0.035	0.41	0.16	0.65	0.45
0.14	0.05	0.04	0.02	0.01	0.11	0.19	0.01	0.31	0.10	0.36	0.15
	Fig. 2, Curve 5	Curve 3		Curve 2		Fig. 2, Curve 1					

$A = 20A'$				$A = 1/5\, A'$					
$r' = 0.9\, r_0$ $D = 0.1\, D'$		$r' = 0.98\, r_0$ $D = 0.1\, D'$		$r' = 0.5\, r_0$ $D = D'$		$r' = 0.9\, r_0$ $D = 0.1\, D'$		$r' = 0.98\, r_0$ $D = 0.01\, D'$	
$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(6.13)	(8.00)	(2.12)	(1.76)	(0.3)	(0.61)	(0.79)	(2.98)	(0.96)	(4.85)
1.00	1.00	1.00	0.95	0.95	0.82	0.99	0.82	0.99	0.98
0.99	0.94	0.97	0.80	0.79	0.40	0.92	0.68	0.93	0.88
0.98	0.91	0.94	0.70	0.58	0.17	0.84	0.58	0.86	0.80
0.97	0.87	0.90	0.60			0.73	0.45	0.77	0.69
0.95	0.82	0.84	0.50			0.56	0.33	0.64	0.55
0.92	0.76	0.76	0.40			0.35	0.19	0.46	0.39
		0.65	0.31					0.23	0.19
		0.59	0.27					0.10	0.08
						Curve 4		Curve 6	

C_s is the largest value of C and hence $F(C_s)$ is the largest value $F(C)$ can have. Replacing $F(C)$ by this constant maximum value and also, in the case $r \neq 0$, replacing r by 0 (this gives a larger range of integration, so that a larger value of the integral is obtained), we have



Curve	$\frac{A}{A'}$	$\frac{D}{D'}$	$\frac{r'}{r_0}$
1	5.0	1.00	0.67
2	1.0	1.00	1.00
3	1.0	0.10	0.98
4	0.2	0.10	0.90
5	1.0	0.01	0.90
6	0.2	0.01	0.98

See text for details.

FIG. 2.

$$C \geqq C_s - \int_r^{r} \frac{1}{r^2}\left(\int_o^{r} r^2 F(C_s)dr\right)dr.$$

And, completing the integration,

$$C \geqq C_s - \frac{F(C_s)}{6}(r_0^2 - r^2). \tag{24}$$

Hence, if $F(C_s)$ is such that

$$F(C_s) < \frac{6C_s}{r_0^2}$$

C is greater than 0 for any r. That is, if $F(C)$ is such a function that

$$F(C_s) < \frac{6C_s}{r_0^2}$$

for a certain range of values of C_s, then in this range C is greater than 0 for $r \geqq 0$ for any C_s. In the further development this inequality will be assumed for the range of C_s employed. A first approximation may be obtained by assuming $F(C) \ll \frac{6C}{r_0^2}$. (See later.) Then from (24) C is approximately equal to C_s. And, as a second approximation,

$$C = C_s - \int_r^{r_0} \frac{1}{r^2} \left(\int_o^r r^2 F(C_s) dr \right) dr$$

$$= C_s - \frac{F(C_s)}{6} (r_0^2 - r^2).$$

Returning to equation (22), the first approximation gives

$$Q = 4\pi D \int_0^{r_0} F(C_s) r^2 dr$$

$$= \frac{4\pi D F(C_s) r_0^3}{3}.$$

That is, for high C_s, $Q = kF(C_s)$, where $k = \frac{4\pi D r_0^3}{3}$. Using the second approximation,

$$Q = 4\pi D \int_o^r F\left[C_0 - \frac{F(C_s)}{6} (r_0^2 - r^2) \right] r^2 dr.$$

Expanding, assuming C_s large compared with $\frac{F(C_s)}{6}(r_0^2 - r^2)$,

$$Q = 4\pi D \int_0^{r_0} \left[F(C_s) - \frac{F(C_s)}{6} (r_0^2 - r^2) \frac{dF(C_s)}{dC_s} \right] r^2 dr$$

$$= \frac{4\pi D r_0^3 F(C_s)}{3} \left[1 - \frac{r_0^2}{15} \frac{dF(C_s)}{dC_s} \right]. \tag{25}$$

This shows that the effect of diffusion (in lowering C inside the cell and therefore A and Q), represented by the term in brackets, is very small unless $\frac{dF(C_s)}{dC_s}$ is very large.

Experimentally, Q is obtained as a function of C_s. Dropping the subscript, s,

$$Q(C) = kF(C) \left[1 - \frac{r_0^2}{15} \frac{dF(C)}{dC} \right]. \tag{25a}$$

Let $F(C) = \frac{Q(C)}{k} + r_0^2 H(C)$ + higher powers of r_0 times functions of

C. Substituting in (25*a*)

$$Q(C) = Q(C) + kr_0^2 H(C) - Q(C)\frac{r_0^2}{15k}\frac{dQ(C)}{dC}$$

plus higher powers of r_0 times functions of C, or

$$H(C) = \frac{Q(C)}{15k^2}\frac{dQ(C)}{dC}\,.$$

Hence

$$\begin{aligned} F(C) &= \frac{Q(C)}{k} + \frac{r_0^2 Q(C)}{15k^2}\frac{dQ(C)}{dC} + + \cdots \\ &= \frac{3Q(C)}{4\pi D r_0^3}\left(1 + \frac{1}{20\pi D r_0}\frac{dQ(C)}{dC}\right) + \cdots. \end{aligned}$$

By Taylor's theorem, neglecting higher powers,

$$F(C) = \frac{Q(C^*)}{k}\,,$$

where

$$C^* = C + \frac{r_0^2}{15k}Q(C);$$

and since

$$A(C) = DF(C),$$

therefore

$$A(C) = \frac{3}{4\pi r_0^3}Q(C^*) \tag{26}$$

and

$$C^* = C + \frac{Q(C)}{20\pi D r_0}\,. \tag{27}$$

It will be noted that the larger the value of D, the more nearly do $Q(C)$ and $Q(C^*)$ agree. Q decreases with decrease of C more rapidly than does the true function $A(C)$. This is due to the factor of diffusion, making C at interior regions of the cell lower than C_s. Obviously the closer C approaches C_s, the more nearly will the observed oxygen consumption of the whole cell equal the theoretical consumption per unit volume where this volume has an oxygen pressure of C_s throughout. The higher the diffusion constant and the smaller the radius, the closer will the approach be. The effect of D is apparent in equations (26) and (27), but an increase of radius, r_0, might at first seem to likewise reduce the diffusion factor. When it is recalled, however, that $Q(C)$, the total consumption of a cell, varies with r_0^3, it is clear that the diffusion effect increases as the square of the radius.

For unfertilized sea urchin eggs, Tang (1931) has obtained the data necessary for the calculations. These indicate that the maximal correction is 0.5 per cent, if the diffusion constant be as assumed. For a constant 100 times smaller, the correction is important, and this case is illustrated in Table III and the resultant $A(C)$ curve shown in Fig. 3. For luminous bacteria, because of the very small r_0, the correction turns out to be entirely negligible and the $Q(C)$ curve as observed is also the $A(C)$ curve (Fig. 3).

TABLE III

For unfertilized *Arbacia* eggs:
$Q_0 = 6.7 \times 10^{-10}$ cc. O_2 per minute, per egg.
$D = 1.1 \times 10^{-7}$ cc. O_2 per minute, per cm.^{2} per diffusion gradient of one atmosphere per cm. (1/100 Krogh's value assumed).
$C_c = 0.1$ atmosphere.
$r_0 = 3.8 \times 10^{-3}$ cm.

1	2	3	4	5	6	7	8
C_S	$\frac{C_S}{C_c}$	$Q(C_S) \times 10^{-10}$	$\frac{Q(C_S)}{Q_0}$	C^*	$Q(C^*) \times 10^{-10}$	$\frac{Q(C^*)}{Q_0}$	$A(C) \times 10^{-3}$
0.100	1.00	6.7	1.00	0.125	6.7	1.00	2.92
0.090	0.90	6.6 +	0.99	0.115	6.7	1.00	2.92
0.065	0.65	6.6 −	0.98	0.090	6.6 +	0.99	2.89
0.050	0.50	6.4	0.96	0.074	6.6	0.98	2.86
0.035	0.35	6.2	0.92	0.057	6.5	0.97	2.83
0.022	0.22	5.7	0.85	0.044	6.3	0.94	2.74
0.010	0.10	4.4	0.66	0.027	5.9	0.88	2.57
0.004	0.04	3.1	0.46	0.016	5.0	0.75	2.19
0.000	0.00	0.0	0.00	0.000	0.0	0.00	0.00

Columns 1 and 3 are taken from the data on *Arbacia* eggs. These

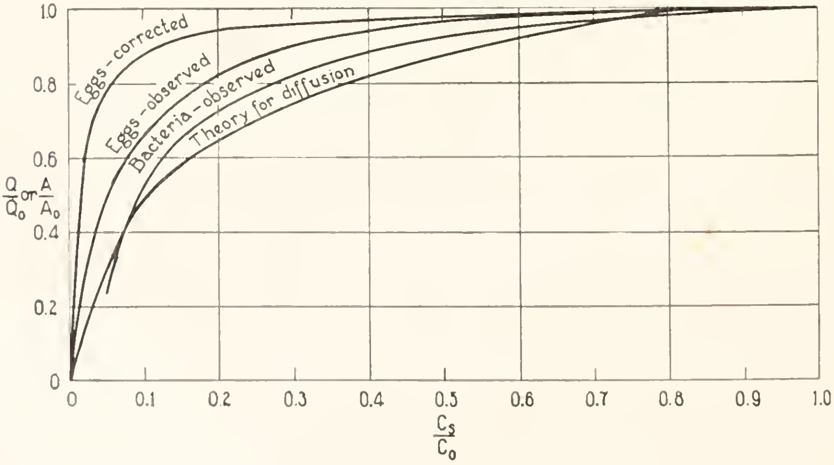


FIG. 3.

values are expressed as ratios in Columns 2 and 4. C^* is calculated by equation (27). $Q(C^*)$ is again obtained by interpolation on the $Q(C)$ curve for eggs, taking Q for each value of C^* as if of C. Column 7 is obtained from 6 and Column 8 from 6 and equation (26). A, as a function of C, is expressed as volume of oxygen per equal volume of eggs per minute.

It may be observed that the assumption made in this last derivation, that $A \ll \frac{6CD}{r_0^2}$ is fully justified for this case. Table IV shows the actual values of each side of the inequality for several values of C.

TABLE IV

C	A	$\frac{6CD}{r_0^2}$
0.1	0.0029	0.480
0.05	0.0029	0.240
0.01	0.0026	0.048
0.004	0.0022	0.019

Examination of Available Data

There are a number of data in the literature for several types of cells sufficiently complete to permit the calculation of the simple C_0; fewer giving as well the observed C_0. Table V summarizes most of this material. It will be noted that the observed C_0 is from 30 to 1000 times greater than the calculated one in all cases except that reported by Warburg and Kubowitz (1929). These workers offer evidence that inadequate shaking at the low oxygen pressures may cause the liquid phase to lag behind the gas phase in oxygen pressure, the dissolved oxygen being used by the cells faster than it can be replaced from the gas phase. Where such a non-equilibrium state obtains, the actual C_0 would be less than supposed, which might account for a reported high C_0. In the experiments of Gerard and Falk (1931), as well as those of Tang (1931), at least, this factor was considered and apparently excluded by controls of the rate of shaking. As pointed out by these workers, wide variation in the conditions of shaking did not affect the C_0. In Amberson's experiments no gas phase was present, only dissolved oxygen being available to the cells from a large volume of liquid. Also, it may be noted that Stephenson and Whetham (1924) found *B. coli* forming lactic acid in air but not in oxygen; and Novy and Soule (1925) observed for the case of tubercle bacilli that growth on the surface of an agar slant was retarded at

TABLE V

Material	Observer	Radius cm. $\times 10^{-4}$	Temp. ° C.	Oxygen consumption cu. mm. per minute		C_0 (Atmosphere)	
				per 10^6	per cu. mm.	Observed	Calculated *
Sarcina lutea	Gerard and Falk, 1931	0.63	20	5×10^{-5} 1.7×10^{-4}	0.05 (H_2O) 0.17 (gluc.)	0.01 0.025	1.2×10^{-5} 3.8×10^{-5}
Sea urchin eggs	Tang, 1931 Amberson,† 1928	38	21 20	0.67 3.3	0.004 (unfert.) 0.022 (fert.)	0.10 0.10	7.4×10^{-4} 2.7×10^{-3}
Luminous bacteria	Shoup, 1929 Harvey, 1928	0.6×1.1	21	5×10^{-4}	0.3	0.03	1.9×10^{-5}
Micrococcus candidans	Warburg and Kubowitz, 1929	0.4	10			0.00005	$\alpha 10^{-5}$
Yeast	Warburg, 1926	3.5	20		0.2 (gluc. and PO_4)	$0.04 <> .20$	4×10^{-5}
Amoeba	Pantin, 1930	50×13	15			0.05	5×10^{-4}‡
Nitrifying bacteria	Meyerhof, 1916	.	35			0.20	
Colpidium	Pitts, (unpublished)	25×15	24	2.5	0.1		6×10^{-3}

* D has been taken throughout as equal to 1.1×10^{-5} (expressed as cc. O_2 diffusing per minute, per cm.^{2} surface per gradient of 1 atmosphere per cm.)

† For fertilized *Arbacia* eggs, Amberson (1928) finds $C_0 = 0.10$ atmospheres. A cannot be obtained from his data, but he informs me that 3 cc. of a settled egg suspension consumed 0.25 cc. O_2 per hour. If the eggs were just in contact, this would correspond to about 1.5 gm. of eggs, and $A = 0.003$ cu. mm. O_2 per cu. mm. eggs per minute. This value is lower than that obtained by Tang by direct measurement for unfertilized *Arbacia* eggs, and less than 1/7 the corresponding one for fertilized eggs. A similar value for A, 0.002, can be calculated from Warburg's data (1908) on *Strongylocentrotus* eggs, if the same cell radius as *Arbacia* and a specific gravity of 1.05 be assumed.

All agree that fertilized eggs respire five to six times as rapidly as resting ones and the figure used here is 5.5 times that of Tang for the resting cells.

‡ Pantin's calculation.

oxygen pressures below 0.06 atmospheres. In Meyerhof's experiments (1916) also, vigorous shaking is mentioned, and Warburg (1926) found the oxygen consumption of yeast to fall with oxygen pressures below 0.2 atmospheres. On the other hand, despite the careful experimental manipulation described by Warburg and Kubowitz, extra oxygen may have entered their system by diffusion out from the measuring column of Brodie's solution. It is possible that the discrepancy between the results of these experimenters and those of all others is to be attributed to the different cells used.

I am aware of only five sets of observations from which the entire C_s-Q curve can be obtained. The older observations of Amberson (1928) on fertilized *Arbacia* eggs fit almost exactly with those of Tang (1931) on unfertilized ones, even the absolute C_0 values agreeing. This is hardly to be expected with a Q_{O_2} five to six times greater for the fertilized than the resting eggs, and if this identity is confirmed when using the same method on both materials, it will be of considerable importance. Actually, the estimated Q_{O_2} for Amberson's fertilized eggs is less than Tang's value for unfertilized ones (see footnote to Table V), so little more can be said at this time. For bacteria, the curve obtained by Shoup (1929) is available. The complete Q-C_s curve was not obtained in the case of *Sarcina* because of the continually changing Q_{O_2} even in air. Meyerhof (1916, 1917) has presented less complete data for the nitrifying bacteria. All these data are summed up in Table VI. The oxygen consumed by each type of cell is expressed as a fraction of the maximum for that cell, and all are aligned against C_s values expressed as fractions of C_0, this being likewise taken as unity for all.

TABLE VI

$\frac{C_s}{C_0}$	$\frac{Q}{Q_0}$			
	Arbacia eggs	Luminous bacteria	Nitrite formers	Nitrate formers
1.00	1.00	1.00	1.00	1.00
0.90	0.99	0.98	0.99	0.98
0.65	0.98	0.95	0.97	0.88
0.50	0.96	0.92	0.92	0.80
0.35	0.92	0.86	0.79	0.71
0.22	0.85	0.74	0.54	0.59
0.10	0.66	0.53	0.24	0.34
0.04	0.46	0.15	(.13)	
0.00	0.00	0.00	0.00	0.00

It is especially interesting to compare these observed curves with those calculated on the various types of assumption. For the luminous bacteria, the observed curve agrees fairly well with the one calculated on the assumption that there is no concentric zoning of the cell and that oxygen consumption is independent of pressure, except as diffusion becomes inadequate. Q falls with C_s, in other words, as would be expected if the only factor involved were the depth of penetration. Still the absolute value of C_0 as measured is far above that expected on the grounds of the diffusion equation. Either, therefore, the diffusion coefficient for oxygen through the bacterial cell is over 1,000 times smaller than that for fascia and muscle,[2] or the fall of Q with C_s is quite independent of any diffusion factor.

In the case of sea urchin eggs, the situation is still more complicated. The observed curve does not fit the simple calculated one. It does fit, however, a curve calculated on the assumption that A is independent of C but including the complication that A is not uniform in all cell regions but is greater in a cortical zone than in the central portion (Curve I, Fig. 2, and Fig. 3). A lower diffusion constant in the cortical zone can not yield this shape of curve.

Again, the absolute value of C_c, 100 times smaller than that calculated, makes it very difficult to interpret the agreement with the curves calculated on a diffusion basis. If oxygen diffuses through sea urchin eggs 100 times more slowly than through muscle, all the results become quantitatively intelligible. But it is more probable that failing diffusion is only a very minor factor, even in these larger eggs, and that the direct fall of A with falling C is the primary one.

Assuming that $A(C)$ is directly given by the $Q(C)$ curve in the case of luminous bacteria and is obtained by correcting for diffusion in the case of *Arbacia* eggs, we are left with an empiric relation between oxygen concentration and rate of oxidations. Shoup (1929) offers a physical interpretation of his results in terms of adsorption of oxygen on or evaporation from surfaces in the cell. He puts Langmuir's (1916) equation for such gas adsorption in the form:

$$k_1 p(1 - \theta) = k_2 \theta$$

where k_1 and k_2 are adsorption and evaporation constants respectively and θ is the fraction of the total surface covered, and indicates that this will fit his curve if θ be assumed to represent the percentage of maximal respiration, *i.e.*, $\frac{A}{A_0}$ (A_0 being the rate of oxidation at oxygen pressure C_c; A the rate at pressure C). Warburg and Kubowitz (1929) also developed a relationship in terms of reaction velocities for oxidation and reduction of an iron catalyst.

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[2] Each bacterial cell, as any small particle, is surrounded by an envelope of fluid which is essentially not stirred. This increases the effective radius of the cell and hinders diffusion. This alone, however, to increase C_0 1,000 times, would require a layer over 300 times the radius of the cell. (See Equation 7.)

A somewhat similar development follows.

It may be safely assumed that oxygen combines with at least one intermediate catalyst before acting upon the substrate. Then, neglecting any details,

$$\begin{gathered} O_2 + \text{Int.} \rightarrow \text{Int.}O_2 \\ \text{Int.}O_2 + \text{Sub.} \rightarrow \text{Sub.}O_2. \end{gathered}$$

For an equilibrium state, the concentration of Int.O_2 must remain constant and the rates of its formation and reduction must be equal. So

$$[O_2][\text{Int.}]k_0 = A = [\text{Int.}O_2][\text{Sub.}]k_r, \tag{28}$$

where A is the velocity of combination of oxygen, or rate of respiration, and k_0 and k_r constants of oxidation and reduction.

In the presence of an excess of substrate, [Sub.] may be assumed to be a constant, k_s, and

$$A = [\text{Int.}O_2]k_rk_s.$$

The maximal rate of respiration, A_0, will be obtained when all Int., Int.$_t$, is in the oxidized state; and the respiration as a fraction of the maximum is then

$$\frac{A}{A_0} = \frac{[\text{Int.}O_2]}{[\text{Int.}_t]}. \tag{29}$$

From (28)

$$\frac{[\text{Int.}]}{[\text{Int.}O_2]} = \frac{k_rk_s}{k_0[O_2]} = \frac{K}{[O_2]}.$$

Adding 1 to each side and inverting,

$$\frac{[\text{Int.}O_2]}{[\text{Int.}] + [\text{Int.}O_2]} = \frac{[O_2]}{K + [O_2]}.$$

But

$$[\text{Int.}] + [\text{Int.}O_2] = [\text{Int.}_t].$$

So that

$$\frac{A}{A_0} = \frac{[O_2]}{K + [O_2]}.^3 \tag{30}$$

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[3] If [Sub.] is not assumed to be constant, the final equation is

$$\frac{A}{A_0} = \frac{[O_2]}{K[\text{Sub.}] + [O_2]}.$$

When [Sub.] is maximum, the effect of $[O_2]$ is greatest; as [Sub.] becomes less, it, rather than $[O_2]$, becomes the critical factor.

But Langmuir's equation may be readily put in an identical form by using the notation $[O_2]$ for p, $\frac{A}{A_0}$ for θ, and K for $\frac{k_2}{k_1}$. Obviously, an agreement between the curve of this equation and that obtained experimentally fails to substantiate either of the assumed mechanisms as the one underlying these oxidations. As a matter of fact, the experimental data do not agree too well with the equation.

Shoup's data (as read from his curve) yield the following:

Per cent O_2	$\frac{A}{A_0}$	K
3.00 and over	1.00	0.00
1.32	0.90	0.14
0.79	0.80	0.20
0.55	0.70	0.23
0.38	0.60	0.25
0.27	0.50	0.27
0.22	0.40	0.33
0.17	0.30	0.40
0.13	0.20	0.52

It is sufficiently obvious that K is even roughly constant only in the middle range.

Tang's data, similarly handled, give:

Per cent O_2	$\frac{Q}{Q_0}$ $\frac{A}{A_0}$	K
10.0 and over	1.00	0.0
9.0	0.99	0.1
6.5	0.94	0.3
5.0	0.87	0.8
3.5	0.78	1.0
2.2	0.66	1.1
1.0	0.49	1.0
0.4	0.27	1.1
0.0	0.00	—

The agreement here is somewhat better. K is approximately constant up to 80 per cent maximal respiration.

For the nitrifying bacteria, the curves of the nitrite and the nitrate formers are widely different, and both are far from agreeing with the derived equation.

It seems to follow, clearly enough, that the $Q-C_s$ curve is far from identical for the various cells, and that the individual form is probably but little dependent on the limits set by diffusion. No one

	NO_2		NO_3	
Per cent O_2	$\frac{Q}{Q_0}$	K	$\frac{Q}{Q_0}$	K
20 and over	1.00	0.0	1.00	0.0
15	0.99 +	0.1	0.92	1.3
10	0.98	0.2	0.80	2.5
7	0.92	0.7	0.72	7.0
4	0.68	1.9	0.57	4.0
2	0.35	3.7	0.34	3.9

simple assumption as to oxidizing mechanism appears able to fit all cases. Additional data for a much greater variety of cells are greatly to be desired, and must precede any more elaborate attempt to theoretically derive the important $A(C)$ curve.

Further important questions concern the change in Q_0 attending changed conditions. The fertilized sea urchin egg, for example, after a tremendous initial burst of oxidation, continues to use oxygen at five to six times the resting rate. Is this to be interpreted in terms of a sudden increase of available Int.? None the less, so far as present data show, there is no change in C_0 or in $A(C)$. *Sarcina*, on the other hand, shows an increase in C_0 approximately in proportion to the increased Q when glucose is added. The complicated relations between lactic acid and Q_0 have been previously discussed (Gerard, 1931). The suddenly increased respiration of muscle, nerve, and other cells associated with activity also comes to mind in this connection.

Finally, it is to be noted that most cells exhibit a considerable "factor of safety" in their rate of energy liberation. The respiration may be very considerably depressed by low oxygen pressures before the lessened energy interferes with the physiological activity of a cell. Thus luminous bacteria do not begin to dim until the oxygen is reduced to 0.25 per cent (Shoup, 1929), when respiration has been reduced to half, and still luminesce at 0.0007 per cent O_2 (Harvey, 1928). *Arbacia* eggs show no disturbance in cleavage at oxygen concentrations over 1.5 per cent (Amberson, 1928) though 10 per cent is required for maximal respiration.

Similar relations are difficult to demonstrate for tissues because of the tremendously greater importance of diffusion in the larger masses. For the case of nerve, it was shown (Gerard, 1927) that the C_0 calculated from the simple diffusion equation, assuming A independent of C, was in good agreement with the observed value. It is probable, however, that more careful examination of this point would reveal some dependence of A on C. Less direct evidence indicates that here, also, conduction is possible when the energy available is but a fraction of the normal amount. (Gerard, 1930).

SUMMARY

The question of diffusion of oxygen into cells, in distinction to tissues, requires examination of several factors not previously considered. The relation between external oxygen pressure and rate of respiration may be complicated by the existence of different diffusion constants and respiration rates in separate cell zones.

Equations are developed covering such cases for two concentric zones in a sphere and relating rate of respiration to oxygen concentration, cell radius, and diffusion coefficient.

When the assumption, made in similar studies for tissues, that consumption in any one region is independent of oxygen pressure if above zero, is avoided, the observed consumption-pressure curve can be corrected for diffusion effects to give the true consumption-pressure relation. Equations for this correction are presented.

Available data on bacteria, marine eggs, and other material, are examined quantitatively. It appears very probable that oxygen consumption in each region is dependent on oxygen concentration up to quite considerable values. The theoretical bases for such a dependence are discussed.

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THE EFFECT OF TEMPERATURE CHANGES UPON THE PULSATIONS OF ISOLATED SCALE MELANOPHORES OF *FUNDULUS HETEROCLITUS*

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When Spaeth (1916*a*) published a method for producing pulsations in the isolated scale melanophores of *Fundulus heteroclitus* he greatly broadened the possible scope of investigations dealing with the direct action of various physical and chemical factors upon such preparations. Recognizing this fact, it was thought that perhaps an investigation of the effects of temperature changes upon such pulsations would help in extending our knowledge of how heat and cold act upon the pigment cells. The melanophores of isolated *Fundulus* scales will respond directly to variations in temperature (Spaeth, 1913; Smith, 1928), heat causing a contraction and cold an expansion of the pigment granules. But beyond these descriptive facts our knowledge does not go. One's interest is, therefore, directed towards ascertaining how these melanophores, when induced to pulsate through the use of Spaeth's method, would react to temperature changes, with the hope of perhaps obtaining information permitting a more exact analysis of this phenomenon.

Briefly, Spaeth's method of producing pulsations is as follows: First the isolated *Fundulus* scales are immersed in N/10 $BaCl_2$ for 5 minutes, in which time their melanophores become punctate; then they are transferred to N/10 NaCl, where within fifteen to twenty minutes after their removal to this solution, the pulsations become evident. These pulsations take the form of periodic migrations of the pigment granules in and out of the processes of the melanophores, the movements of the individual pigment granules being readily seen with ordinary microscopic magnification. Hence, we have a preparation which, in its rhythmical activity, is comparable to the behavior seen in other effector organs such as cilia, heart muscle, and smooth muscle. To this add the advantage that in the melanophore we deal with an effector consisting of only a single cell. In studying the effects of temperature changes upon pulsating pigment cells, our interest is therefore centered on how this factor may alter either the rate or the extent of these rhythmical migrations, distal and proximal, of the pigment.

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Now it is well known that the color changes exhibited by numerous fishes in response to variations in the tint of the background over which they swim are determined in a large measure by the behavior of these melanophores and allied pigment cells. However, in the intact fish the melanophores do not pulsate; such alterations as take place in the distribution of their pigment are relatively slow in occurrence and only in response to a change in the color of the background or of the incident light intensity. Therefore, in dealing with pulsating isolated melanophores, we are not concerned with a phenomenon which plays a part in the natural economy of the animal.

As a cell the melanophore is a relatively large structure with numerous branching processes exceeding in length by three or four times the diameter of the central body from which they ramify. These processes, when empty of pigment, are transparent under ordinary conditions of illumination. In fact, the visible manifestation of the shape of the cell is due entirely to the pigment granules contained within itself, and if this pigment is concentrated in the central body, the cell appears to be spherical. The term punctate is applied to melanophores in this state. But when under the proper circumstances these invisible processes are invaded by a distal migration of the pigment granules, their outlines are rendered visible. It is this migration of the pigment in and out of the otherwise transparent processes that creates the illusion of a change in the shape of the melanophore. The use of the terms "contraction" and "expansion" of the melanophores must, therefore, be considered as only convenient expressions describing a redistribution of the pigment granules within the melanophores.

An investigation of this nature requires an apparatus sensitive enough to accurately measure these pulsatory movements within the processes of the melanophores. Such an apparatus was modeled with some modifications after one originally devised by Spaeth (1916*b*) for the same purpose, and employed by him in connection with his researches upon the effects of electrical stimulation upon the isolated melanophore. As my investigations were not concerned with the effects of electrical stimulation, but were rather studies of the effects of temperature variations, certain changes were necessarily introduced in the original design, which is fully described and pictured elsewhere (Spaeth, 1916*c*); the most important among these changes being the addition of a warm stage to the set-up. This was done by fitting to the stage of the microscope a water bath in which a small Stender dish could be almost completely submerged. This Stender dish was filled with N/10 NaCl, and in this solution the scales containing the pulsating melanophores were placed. By altering the temperature of the water flowing through the bath, it was also possible to change the temperature of the NaCl. Any desired temperature between 0° and 45° C. could be attained within two minutes in the Stender dish and maintained indefinitely with no variation greater than 0.2° C.

To measure the pulsations and to make a permanent record of their variations, the microscope was equipped with an adjustable micrometer ocular in place of the usual eye piece, the adjusting screw of the ocular being fitted to a pulley operating a lever capable of writing upon a kymograph drum. In making a record, a scale was placed under the objective, and one of its pulsating melanophores selected for observation, a convenient division of the ocular scale being brought level and at right angles to the apparent end of one of the pulsating processes of this selected melanophore. As the pigment granules in this process migrated either distally or proximally, the ocular scale division kept pace with those granules which marked the apparent distal boundary of the process. Such action was accomplished by turning the adjusting screw of the movable ocular scale. To insure accurate measurements, it was absolutely necessary that the melanophore be kept motionless throughout the experiment. This was accomplished first by bringing a microscope clamp, attached to the warm stage, to bear lightly upon the end of the scale, care being taken that the melanophores were not damaged or stimulated by too great a pressure. Since such a contingency was always imminent, this method for holding the scale was eventually discarded in favor of another. The new method consisted in laying a paraffine mould on the bottom of the Stender dish cut so as to accommodate the scale. In this way the scale was snugly secured without being subjected to any noticeable pressure. No effect of the paraffine upon the pulsations could be demonstrated. As the adjusting screw was turned to follow the migrations of the distal pigment granules, the pulley attached to it moved the lever in contact with the kymograph drum up and down. Consequently, with the lever writing upon the smoked kymograph paper, a permanent and accurate record of the pulsations could be obtained. The speed of the drum was adjusted to one revolution in approximately forty-five minutes.

Records of the melanophore pulsations were not made until the cells had been exposed to NaCl for at least one hour subsequent to their immersion in $BaCl_2$. While pulsations are first evident in fifteen to twenty minutes after the removal of the scales from $BaCl_2$, it usually requires an additional thirty minutes before they become constant in extent and frequency at any given temperature. The first pulsations are extremely slight, but once started they gradually become more pronounced until they finally reach the maximum characteristic for the particular temperature in question. An hour, then, gives ample time to insure the attainment of this point. However, once this maximum was reached, a new state of equilibrium incident upon a change in temperature could be attained within 5 minutes. Therefore, a record having been made at a certain temperature, 10 minutes being usually sufficient for this purpose, another record at another temperature could be made 10 minutes after the change in temperature was effected. The extent of the temperature change between observations was from 1° to 10° C., the usual variation being about 5° C. The experiments were begun at either end of the temperature scale, and by a series of four or five steps, the temperature was raised or lowered to the opposite extreme, the result being the same regardless of whether the initial record was made at a high or a low temperature. In this way one melanophore was kept under continuous observation for two or three hours. At the beginning, a single process of a single melanophore was selected, and the record made from the pulsations of this process. The numerous melanophores of a single isolated scale vary considerably in size. Though an attempt was made to confine the experiments to melanophores approximately equal in size, this was not always possible. However, such attempts were more or less nullified by the fact that it was impossible to predict the maximal length of a process by its appearance when the cell was in an almost punctate or even semi-expanded condition. As fully one-half the experiments were begun under such circumstances, certain corrections in the data were necessary before the results could be subjected to satisfactory analysis.

Temperature Changes and their Effect upon the Extent of the Pulsations

On analysing the results obtained from the records pertaining to the relationship between the temperature and the amount of extension of the melanophore processes during their pulsations, two general facts emerge: (1) An increase in temperature causes a decrease in the extent of the pulsations and (2) this relationship between temperature and extension is a rectilinear one. Table I presents the data relative to this relationship. The figures given in the temperature columns represent the average of the mean maximal and minimal lengths of a single selected melanophore process during its pulsations at a given temperature, obtained by measuring in centimeters the height of the highest and lowest points of the curve traced on the kymograph records. This averaging was necessary for two reasons: first because during the pulsations the maximal extensions of a single process were not

TABLE I

Average Migration

C.	9.5°	12°	13°	13.5°	14°	14.5°	15°	15.5°	16°	16.5°	17°	17.5°	18°	18.5°	19°	19.5°	20°	20.5°	21°	21.5°	22°
1				11.5												8					
2							38.5					34.5			30						
3	38	38					32		25.5		13.5										
4											34					25					
5																		16			
6												32									
7																					
8											28						25				
9																					
10																				27	
11									11								7				
12							25.5								18.5						
13			34										23								
14							25										9				
15									37												
16									18.5											11	
17								21									18.5				
Av.							30.2		23		25						15.0				

TABLE I (*Continued*)

Average Migration

C.	22.5°	23°	23.5°	24°	24.5°	25°	25.5°	26°	26.5°	27°	27.5°	28°	28.5°	29°	29.5°	30°	30.5°	31°	31.5°	32°
1							4								1					
2	16.5							10												
3																				
4	17				12															
5	13					11.5					6.5									
6					23								8.5						1.5	
7	39			27				17				6.5								0
8		17.5						6.5						1						
9	23			18			18				8				5			1.5		
10				23					18							8.5				
11		9										2								
12		14.5						10						6						
13	14						12								8					
14	6.5							4.5												
15						19		12			8.5									
16						5.5										1				
17				13					3.5					.5						
Av.	18.4	14.0		20.0		12.0	11.3	10.0			7.7			2.5	4.7					

always constant at a given temperature, a fact which applies equally to the minimal extensions, and secondly because at a point two or three degrees above the lowest temperature at which pulsations occurred (about 12° C.), the process would attain during its period of greatest extension its maximal possible length. However, over these same low temperatures the length of the process during the phase of minimal extension was greater, the lower the temperature, the minimal extension gradually approaching the maximal extension as the temperature came nearer to the critical point. Similarily, as the temperature rose to within two or three degrees of the upper limit (about 32° C.) at which pulsations stopped, the melanophores would appear punctate during their phases of minimal contraction, though at these temperatures the process would keep on periodically extending; the amount of extension, however, decreasing as the temperature rose. On reaching the upper limit all pulsations ceased and the cells remained permanently punctate. Therefore, by using averages of the maximal and minimal lengths, figures are obtained which at the extreme temperatures vary in accordance with the temperature changes. This, of course, necessitates treating the data obtained in the middle of the temperature scale in the same manner.

As indicated in Table I, whenever observations were made upon three or more different processes at the same temperatures, an average of the figures expressing the relative amount of extension was made. These averages are given in the bottom line of the table and are plotted with the result shown in Fig. 1. From this curve it is clear that as the temperature increases, the extension of the melanophore processes during their pulsations decreases; a result to be expected, since it was previously known that high temperatures, when they affect the melanophores directly, cause a withdrawal of the pigment into the central body. Furthermore, it is quite clear from Fig. 1 that the relationship between temperature and extension is a rectilinear one.

The observational data given in Table I represent the true relative lengths of the melanophore processes as they were calculated from the kymograph records. It is apparent, from an inspection of the table, that there is considerable variation in the pulsatory activity among the different processes in regard to the amount of extension shown at the same temperature. Because of this variation, the figures cannot be profitably compared unless they are all corrected on the assumption that the amount of extension in any one process at 20° C. is equal to twenty. When this is done and the results plotted as shown in Fig. 2, it is obvious that the effect of temperature changes on all pulsating melanophore processes is virtually the same so far as any relative alteration in the amount of extension is concerned. The one exception is case 3 of Table I, where the corrected figures were so at variance with the others that it was impossible to plot them upon the curve. They have consequently been omitted from Fig. 2. But aside from this unexplained discrepancy, the curve substantiates the conclusions drawn from the one given in Fig. 1.

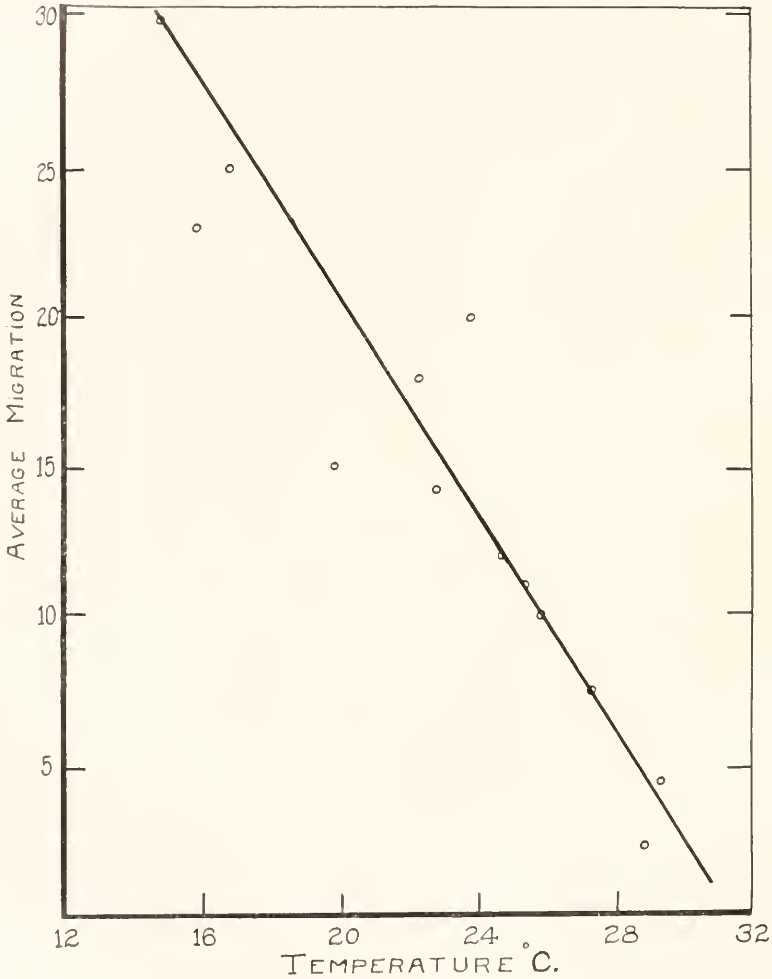

Fig. 1. Showing the rectilinear relationship between the temperature of the medium (abscissæ) and the average length of a melanophore process during a single pulsation (ordinates). The points plotted are those given in the average column of Table I.

In the higher reaches of the temperature scale pulsations cease between 30° and 34° C., the average being approximately 32° C. When this occurs, the melanophore assumes a permanent punctate state. At low temperatures, however, the melanophore comes to rest with all of its processes maximally extended, the pigment being evenly, if thinly, distributed throughout the whole length of the process. Such a condition is generally attained at about 12° C., slight move-

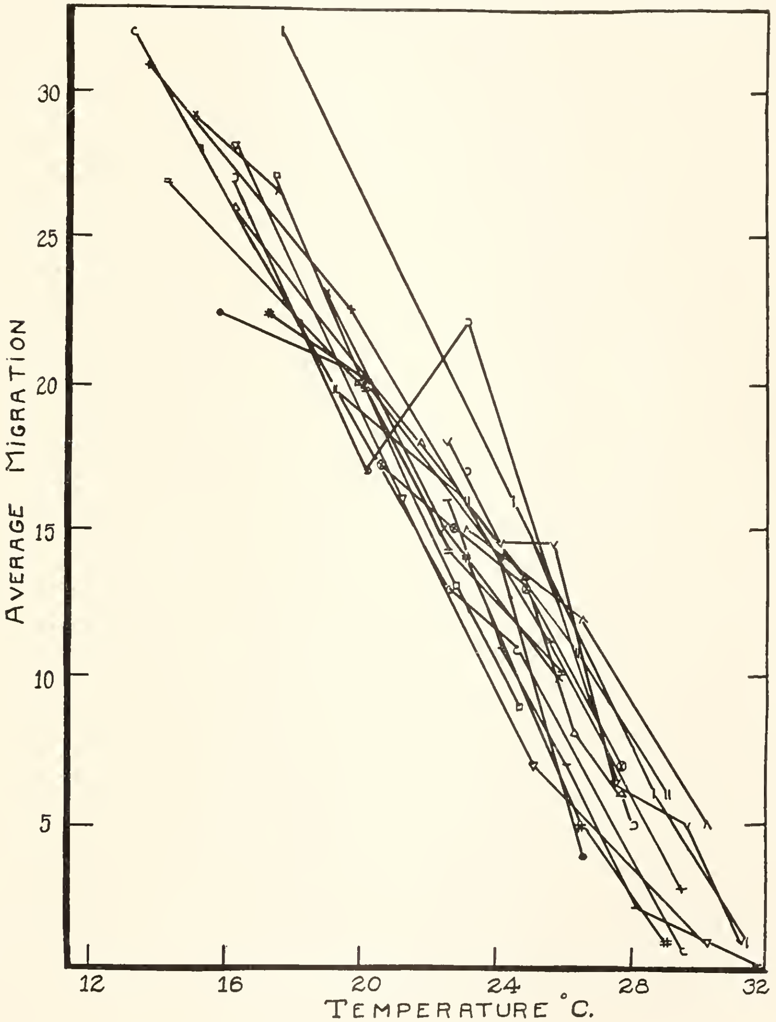

FIG. 2. Showing the result of plotting seventeen different experiments on the relationship between the temperature of the medium (abscissæ) and the average length of the melanophore process during a single pulsation (ordinates); the average length of the melanophore process being taken in all cases as equal to 20 at 20° C., the data in Table I being corrected accordingly.

TABLE II

Pulsations per Minute

C.	9.5°	12°	13°	13.5°	14°	14.5°	15°	15.5°	16°	16.5°	17°	17.5°	18°	18.5°	19°	19.5°	20°	20.5°	21°	21.5°	22°
1				.6												1.8					
2							.7					.52			.85						
3	.5						.9		1.1		1.35										
4											.8					1.2					
5																		.4			
6												.4									
7																					
8											.8						.9				
9																					
10																				1.3	
11									.8								1.2				
12							.8								1.5						
13			.2										.4								
14							.4										.9				
15									.8												
16									1.3											1.8	
17								.3									.4				
Av.							.7		1.0		.98						.85				

TABLE II (*Continued*)

Pulsations per Minute

C.	22.5°	23°	23.5°	24°	24.5°	25°	25.5°	26°	26.5°	27°	27.5°	28°	28.5°	29°	29.5°	30°	30.5°	31°	31.5°	32°
1							2.5								3.0					
2	1.4							2.0												
3																				
4	1.9				2.1															
5	.7					1.1					1.8									
6					.6								1.7						1.1	
7	1.1			1.3				1.7				2.4								0
8		1.0						1.5						1.2						
9	1.2			2.1			1.8				2.1				2.2			1.8		
10				1.6					1.8							2.3				
11		2.1										5.0								
12		4.0						4.4						1.1						
13	.9						1.3								2.4					
14	1.2							2.4												
15						2.0		2.8			3.4									
16						3.7										8.0				
17				1.0					1.4					.8						
Av.	1.2	2.3		1.5		2.27	1.87	2.46			2.43			1.03	2.53					

ments persisting in some cells until 10° C.; while others come to rest at temperatures as high as 14° C. In many cases Brownian movement was observed among the pigment granules in all parts of the process, even though the pulsations had entirely ceased.

TEMPERATURE CHANGES AND THEIR EFFECT UPON THE FREQUENCY OF PULSATIONS

On considering the action of temperature changes upon the frequency of pulsations, we find (1) that an increase in temperature from 12° C. to within the neighborhood of 27° C. causes an increase in the rate of pulsations, which is followed by a rapid decrease as the temperature mounts higher, and (2) that the relationship between temperature and the increase in rate during the rise from 12° to about 27° C. is a non-rectilinear one. The data obtained from an analysis of the kyomograph records are presented in Table II, where the figures given in the columns under the temperature headings show the absolute number of pulsations of a single melanophore process in one minute at the temperature indicated. Every figure in the temperature columns represents the pulsations per minute of a different process, no two processes being attached to the same melanophore and every melanophore being selected from a different scale. Between different cells the variation in the frequency of the pulsations at the same temperature is considerable, as shown in Table II; at 22.5° C., for example, a process of one cell shows 0.7 pulsation per minute, while a process of another cell shows 1.9 pulsations per minute. In every case where three or more observations were made at the same temperature, the results were averaged, the averages being given in the bottom line of the table.

In Fig. 3 these averages are plotted, such plotting bringing out the previously-mentioned increase in rate of pulsation during the greater part of the temperature rise, the relationship in this case, in contrast to the relationship between temperature changes and the extent of the pulsations, being clearly non-rectilinear in nature. The possible significance of this will be discussed later. From this curve the temperature coefficient of the melanophore pulsations can be calculated, though such coefficients can have only an approximate value. Between 15° C. and 25° C. the temperature coefficient is 3.57, while between 20° C. and 30° C. it is 3.6. It is doubtful whether the difference between the two is significant, especially as the latter coefficient can have only a speculative value, owing to the irregular appearance of the depressant effect upon the rate of pulsations at the warmer end of the temperature scale.

As there is a wide variation in the behavior of different processes at the same temperature, the data presented in Table II must be corrected before they can be presented in intelligible graphic form. In doing this it was arbitrarily assumed that the rate of pulsation of all melanophore processes at 20° C. was equal to one, the necessary corrections for all other temperatures being made on this basis. These corrected data were then plotted as shown in Fig. 4, the curve so obtained substantiating the relationship expressed in Fig. 3. Furthermore, the points of Fig. 4 group themselves closely about the curve given in Fig. 3.

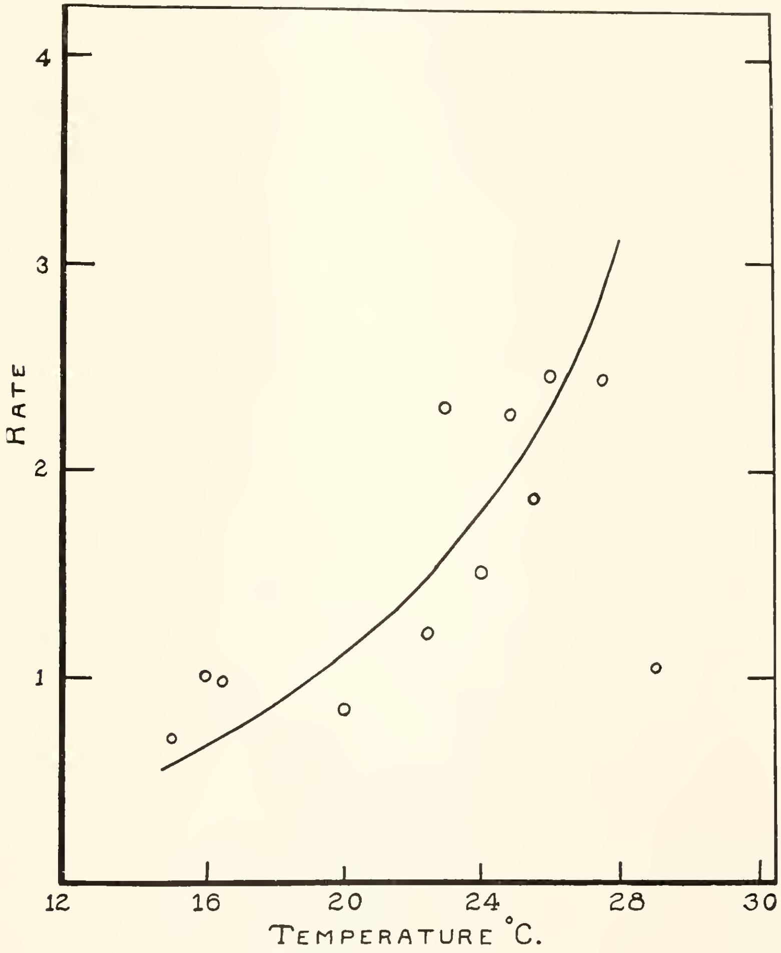

FIG. 3. Showing the non-rectilinear relationship between the temperature of the medium (abscissæ) and the number of pulsations per minute (ordinates). The points plotted are those given in the average column of Table II.

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As to the point where the frequency of pulsations decreases with further increases in temperature, there is considerable variation among the different melanophores, some showing no diminution in the increase in rate at temperatures as high as 30° C., while in others this depressant effect is initiated at temperatures as low as 25° C. In general, this point occurs in the neighborhood of 27° C. Once a

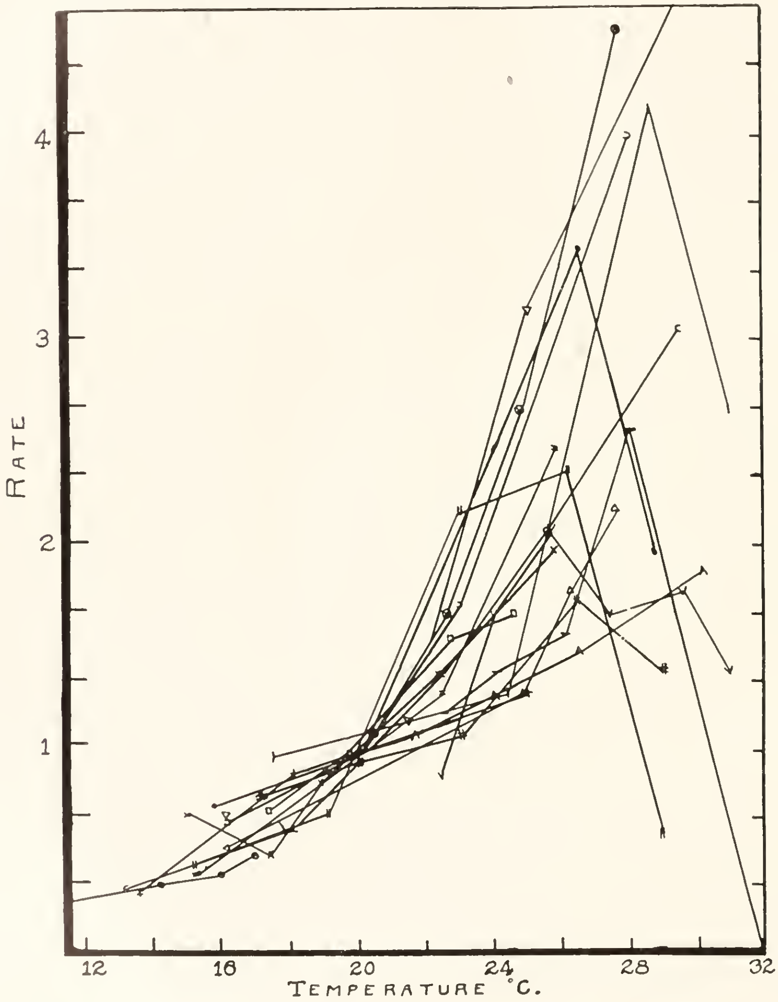

FIG. 4. Showing the result of plotting seventeen different experiments on the relationship between the temperature of the medium (abscissæ) and the number of pulsations per minute (ordinates); the number of pulsations per minute being taken in all cases as equal to one at 20° C., and the data in Table II corrected accordingly.

decrement in rate is established, a further rise in temperature produces a fall, which is very rapid in comparison to the relatively slow increment which preceded it, the difference being graphically shown in Fig. 4. The exact course of this reduction in rate is uncertain, as the data at hand do not permit an accurate analysis.

Discussion

A rise in temperature, then, affects the pulsating pigment granules of an isolated *Fundulus* scale melanophore by increasing the frequency of their pulsatory movements up to a certain point and at the same time reducing the distance migrated by these granules during their pulsations, which in effect gives the appearance of a shortening of the melanophore process. Gray (1923) mentioned a somewhat similar situation in his study of the action of temperature changes upon ciliary activity when he observed a progressive reduction in the amplitude of the ciliary beat between 34° and 38° C., though the frequency of the beat increased in proportion to the rise in temperature. In Gray's experiments, however, this reduction in amplitude was seen only above 34° C. Between 0° and 34° C. the amplitude of the beat remained constant, though the frequency increased as the temperature became higher. One must, therefore, make a distinction between cilia and melanophores in this respect, for in the latter any temperature rise capable of increasing the frequency of the pulsation also produces a corresponding decrease in the extension of the granules during these pulsations. Gray suggests that in his experiments the decrease in the amplitude of the ciliary beat above 34° C. might be explained by assuming "that at this temperature the rate at which the process of contraction is induced in the cilium is more rapid than the process of relaxation, so that relaxation begins before contraction is complete."

The decrease in apparent extension of the melanophore process with an increase in temperature might also be explained in the same way. Once a distal migration of the pigment granules has been established, there is the possibility of its continuing until either factors are brought into play, producing a movement in the opposite direction, or until the granules reach the end of the process and are incapable of further migration. But except with extreme low temperatures, proximal pigment migration sets in before the limits of distal migration are reached. Therefore, the factors producing this proximal migration are capable of overcoming those governing the distal migration, or else the latter cease to operate before the pigment granules reach the limits of the process. But whichever explanation is correct, the matter is of secondary importance, as the factors determining the extent of the migration of the pigment granules must be independent of both. This conclusion is deducible from the response of melanophore pulsations to variations in temperature, for such variations seem to influence independently two separate series of reactions within the pigment cell, one governing frequency, and the other the extent of the migration. The fact that the distance migrated by the pigment granules during their pulsations shows a rectilinear relationship while the frequency of the pulsations varies in a non-rectilinear manner in relation to temperature changes hardly warrants any other interpretation. Therefore, if extension is determined by frequency, and it is by no means clear that this is so, it is only because the factors leading to a proximal migration dominate those which might favor further extension and not because extension is a function of frequency. It is interesting to note in this connection that Clark (1920) demonstrated in the isolated rabbit's auricle a rectilinear decrease in the height of contraction with an increase in temperature; and at the same time observed a non-rectilinear increase in the frequency of the beat. In isolated frog's heart this was not the case, both the diminution in the force of contraction and the increase in frequency of the beat caused by a rise in temperature being of a non-rectilinear nature.

Since melanophores contract in the presence of an insufficient amount of oxygen, there may exist a relationship between the decrease in the oxygen tension of the water with an increasing temperature and the decrease in extension of the melanophore processes. But Spaeth (1913) has shown that in the isolated scale, melanophore contraction of the pigment occurs at high temperatures both in media furnished with an excess supply of oxygen and in media with the usual oxygen tension for the temperature in question. Therefore, the contraction of the pigment granules with an increase in temperature cannot be due to a lack of oxygen. But the contraction of pigment in a non-pulsating melanophore may be governed by factors other than those which control the amount of extension of the pigment granules in a pulsating melanophore. Unfortunately this question is left open as my observations did not include the effect of a decrease in oxygen supply upon either the frequency or the extent of pulsations and nothing is known concerning this question from other sources. The sudden decrease in the frequency of pulsations with temperatures above 27° C., following a consistent increase with a rise in temperature to this point, may be concerned with a possible inadequacy in the oxygen supply to the melanophores at high temperatures. Whether or not this is true for pulsating melanophores, it is apparently untrue in the case of cilia, for, according to Gray (1923) the oxygen consumption of the tissue keeps pace with the speed of the beat over the temperatures used in his experiment. As a consequence of this, Gray doubts whether a rise in temperature involves in well-ventilated tissues a lack of oxygen except with very high temperatures, when a decrease in mechanical activity might well be due to insufficient oxygen.

The effects of temperature changes upon pulsating melanophores and the effects of temperature changes upon ameboid movement, ciliary activity, and heart beat show a certain similarity. In all cases, the effect is one of an increase in frequency, an increase which is more rapid than a rectilinear function. But while the temperature coefficient of ameboid movement, ciliary activity, and heart beat are all in fairly close agreement, the melanophore is peculiar in that it has a temperature coefficient which in comparison to the others is remarkably high. As we have previously seen, the temperature coefficient of a pulsating melanophore is 3.57 between 15° and 25° C., and 3.6 between 20° and 30° C., figures which can be compared with those given in Table III.

TABLE III

Q_{10}	15°–25° C.	20°–30° C.
Ciliary activity	2.15	1.95 (Gray, 1923)
Ameboid movement	2.04	—— (Pantin, 1924*b*)
Heart beat	2.10	1.90 (Clark, 1920)
Melanophore pulsations	3.57	3.60

A determinative rôle on the part of viscosity changes has already been assigned as a possible factor controlling the movements of pigment granules within the melanophores and it is easy to imagine the same factor assuming considerable importance in governing the frequency and the extent of melanophore pulsations. But unfortunately our knowledge of the exact effect of temperature changes upon the viscosity of protoplasm is at present too meager and too contradictory to warrant any extended conclusions as to how much viscosity is involved in responses of melanophore pulsations to variations in temperature. Both Pantin (1924*a*) and Heilbrunn (1924) have interested themselves in the relationship between temperature and its effect upon animal protoplasm, but with no agreement as to results. Pantin, who worked with the protoplasm of *Nereis* eggs, states that as the temperature increases, the viscosity decreases, the relationship being a non-rectilinear one with a temperature coefficient of 1.3 between 15° C. and 25° C. and 1.26 between 20° C. and 30° C. Heilbrunn, on the other hand, working with *Cumingia* eggs found a sharp decrease in viscosity between 0° C. and 2° C., followed, as the temperature rose further, by a gradual increase which came to a maximum in the neighborhood of

16° C. This in turn was replaced by a decrease until the temperature reached about 30° C., when within the next degree or two there was a very rapid increase. But on the basis of either Pantin's or Heilbrunn's work it is clear that viscosity is not an important factor in determining the responses of melanophore pulsations to temperature changes. The difference between the temperature coefficients which Pantin obtained and those found to apply to melanophore pulsations are so great that the viscosity changes in the melanophores can hardly be of importance in governing the variations in pulsations associated with temperature changes. While, on the basis of Heilbrunn's results, if viscosity changes within the melanophore were an important factor, the curves obtained in these studies on the relationship between temperature changes and the amount of extension of the pigment granules together with the frequency of their pulsations would hardly be as regular as they are, but would show significant variations or breaks in accordance with the curve obtained by Heilbrunn. For the present at least, the conclusion seems inevitable that viscosity changes in the protoplasm of the melanophores attendant upon variations in temperature are not responsible for the particular manner in which the pulsations of these cells respond to alterations in temperature. Whether viscosity is of importance in governing the non-pulsatory changes which occur in the distribution of melanophore pigment, as a fish adapts itself to shifts in the color of its background, is quite possibly another matter and one about which nothing can be stated here.

SUMMARY

1. Pulsations in isolated scale melanophores of *Fundulus heteroclitus* occur in N/10 NaCl after treatment with N/10 $BaCl_2$ at any temperature between 12° and 32° C.

2. Below 12° C. pulsations cease, the melanophore coming to rest in the maximally expanded state.

3. Above 32° C. pulsations also cease, the melanophore coming to rest in the punctate state.

4. Between 12° and 32° C. the extent of the pulsations decreases in a rectilinear manner with an increase in temperature.

5. Between 12° and 27° C. the rate of pulsations increases in a non-rectilinear manner with an increase in temperature. With a further rise in temperature above 27° C. there is a rapid decrease in the rate of pulsations.

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INFLUENCE OF CYANIDE AND LACK OF OXYGEN ON THE ACTIVATION OF STARFISH EGGS BY ACID, HEAT AND HYPERTONIC SEA-WATER

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In previous papers (Lillie, 1915, 1917, 1926) I have called attention to the general resemblances between the phenomena of acid activation and of heat activation in unfertilized starfish eggs,—a parallelism indicating that both forms of activation are to be referred to the same kind of change in the egg system.[1] This primary or critical change in some way transforms the egg from the quiescent into the automatically developing or "activated" state. Since exposure to temperatures above the physiological range is known to produce acid (especially lactic acid) in many cells (as seen, *e.g.*, in the phenomena of heat rigor), the inference is natural that heat activation is in reality a secondary effect resulting from the production of acid within the egg; and the essential problem becomes that of determining the factors concerned in acid activation. Experiments on the relative activating effectiveness of a variety of weak penetrating acids having a wide range of dissociation constants (Lillie, 1926 and 1927) indicate that the essential factors in a given activating effect are (1) the production of a certain definite degree of acidity in some region of the egg (probably cortical) and (2) the maintenance of this acidity for a definite time. Some progressive change, the "activation process" or "activation reaction," occurs within the egg during this time; and if the egg is to be rendered capable of developing to an advanced stage, the process must advance to a certain stage of completion. One of the most striking features of the activation process in starfish eggs is that it is readily arrested at any stage by simply returning the eggs to normal sea-water; in such a case it may be renewed and brought to completion by a second exposure to heat or acid, applied after not too long an interval (Lillie, 1915, pp. 284 *seq.*). The rate of the activation process, in the case of a particular acid, appears closely proportional to the concentration of the latter in the external medium—more specifically to the concentration of its undissociated molecules, which alone appear to penetrate freely to the site of the activation reaction (Lillie, 1926 and 1927).

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[1] The chief parallels are (1) the similarity in the time-relations of partial and complete activation by the two methods; (2) the fact that both are completely effective only during the premataurational period of the egg; (3) the substitutability of one for the other in experiments on partial activation, and (4) other parallels described in the present paper.

Since apparently any non-toxic penetrating acid can activate starfish eggs, the special chemical nature of the acid would seem to be immaterial; its presence merely enables the activation process to proceed in the egg system. In general our present data indicate that the rate of activation is closely proportional to the acidity (cH above a certain critical level) attained at the site of activation. The most reasonable assumption appears to be that activation is an effect of the chemical union of certain specific substances already present or available in the egg,[2] and that this interaction, the primary or key reaction in the activating sequence, occurs only above a certain level of acidity (*i.e.*, within a certain pH range). The implication is that the product of this reaction—which we may call the "activating substance"—requires to accumulate to a certain definite level if complete activation is to result. Eggs in which this critical quantity of activating substance has been formed proceed normally with their development if placed under appropriate external conditions. That the accumulation of some material is a necessary condition of activation is indicated by the progressive character of the activation reaction, and especially by the possibility (already referred to) of arresting it at an incomplete stage and afterwards renewing it.

The activation reaction cannot be characterized definitely in chemical terms at present. The fact that responsiveness to activation, either by parthenogenetic agents or sperm, begins normally to decline at or about the time of separation of the first polar body and remains incomplete during the post-maturational period indicates a loss or destruction of some reactant or reactants at this time.[3] Completely mature eggs are capable of only partial activation: although such eggs still form fertilization-membranes and cleave after treatment with fatty acids or warm sea-water, only a small proportion (if any) develop to swimming stages; similarly only a minority of eggs fertilized with sperm during this period exhibit normal development. The capacity for complete activation is lost, apparently permanently, during the progress of the maturation divisions (Lillie, 1908).

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[2] This is also the assumption of the fertilizin hypothesis of F. R. Lillie, which is quite consistent with the present view. *Cf.* Lillie, F. R.: "Problems of Fertilization," University of Chicago Press, 1919; also E. E. Just: "The Present Status of the Fertilizin Theory of Fertilization." *Protoplasma*, 1930, Vol. 10, p. 300.

[3] The term "prematurational period" is used in the present paper to designate the period between the dissolution of the germinal vesicle and the separation of the first polar body; the post-maturational period is that succeeding the separation of the second polar body.

This change in the physiological properties of the egg, like the change leading to its natural death if it is left unfertilized, (Loeb, 1902 *a* and *b*) is intimately dependent on normal respiration, as is shown experimentally by the effects of cyanide or deprivation of oxygen. If freshly removed starfish eggs are placed for two to four hours (at 20°) in sea-water freed of oxygen or containing KCN (M/500, M/1000), and are then returned to normal sea-water, they show an almost unimpaired response to fertilization or artificial activation; while the response of control eggs left for the same interval in normal sea-water is partial or defective. Such facts indicate that some material essential to the activation reaction (*i.e.*, some precursor or precursors) is removed or destroyed at a definite stage in the normal oxidative metabolism of the egg; apparently this occurs most rapidly during the period at which the polar bodies are being separated.

A further fact of significance is that the activation reaction, as such (*i.e.*, the reaction occurring during the actual exposure to fatty acid or heat), is independent of immediate oxygen consumption; that is, the eggs respond in a normal manner to heat or fatty acid while immersed in oxygen-free or cyanide-containing sea-water—even if they have previously been exposed for some hours to these media. Such suppression of oxidative metabolism has, however, a characteristic modifying influence on the rate of activation, as is shown by the experiments to be described below.

1. *Influence of Oxygen-lack or Cyanide on the Effect of a Second Activating Treatment Following Partial Activation by a First Treatment*

It was found in earlier experiments (Lillie, 1915, pp. 284 *seq.*) that two successive brief exposures to either heat or fatty acid, separated by a considerable interval (up to 30 minutes or more), may by a process of summation result in complete activation, although either exposure acting alone has only a partial effect.[4] A single continuous exposure lasting as long as the sum of the other two also causes complete activation. Such an experiment shows that the modification (whatever its nature) produced by the first exposure persists for some time, and that the second exposure induces further modification of the same kind. If the first exposure is made early, *e.g.*, within 5 to 10 minutes after the commencing dissolution of the germinal vesicle, an hour or more may elapse without marked decline in the effect of the second exposure; later this exposure becomes progressively less and less effective. This decline in the response to a second activating treatment appears to follow the same course as the normal decline of activability already described. It also depends on the oxidative metabolism of the egg and can be greatly retarded by cyanide or removal of oxygen; apparently it is referable to a depletion of the reserve of activable material (precursor or precursors of the activating substance) left unchanged by the first treatment.

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[4] In partial activation the eggs form typical fertilization-membranes, but cleave slowly and irregularly (if at all) and die before reaching the blastula stage. In complete activation cleavage approaches the normal in rate and regularity and the great majority (in favorable cases all) of the eggs form active blastulæ and gastrulæ. All gradations in degree of activation can be obtained by varying the length of exposure.

The following experiment (June 10, 1927) will illustrate. The mixed eggs from three starfish were exposed during the prematurational period for 6 minutes to .002 M acetic acid in balanced NaCl$-$CaCl$_2$ solution at 20° C. Part (*A*) was then transferred to normal sea-water (at *ca.* 18° C.) and part (*B*) to sea-water containing M/1000 KCN. At the intervals indicated in Table I eggs from both lots were again exposed for 6 minutes to the same acid solution and returned to normal sea-water. The total exposure, 12 minutes, is approximately the optimum for this solution at 20°. The intervals between the two successive exposures varied from 11 minutes to two hours. The results are shown in Table I.

TABLE I

Interval between exposures (*minutes*)	Percentage of eggs forming blastulæ as result of second exposure	
	A. Eggs kept in normal sea-water	B. Eggs kept in M/1000 KCN in sea-water
11	90	90
20	60–65	90
30	60–70	90
45	60–70	70–80
60	60–70	80–90
90	50	70–80
120	5	50

Eggs exposed for 6 minutes without any second treatment showed typical partial activation, and a few (2–3 per cent) formed blastulæ. Sperm-fertilized eggs developed normally.

In normal sea-water nearly all the eggs have lost responsiveness to the second treatment after two hours, while of the eggs remaining in cyanide-containing sea-water for the same interval a large proportion developed. Table II describes a second similar experiment (July 11, 1927) with longer intervals between exposures.

TABLE II

July 11, 1927. The procedure was the same as in the previous experiment. The acid solution used was .0015 M acetic acid in Na–Ca solution at 20° C. A control series showed an optimum continuous exposure of 12–14 minutes, yielding 60–70 per cent blastulæ. The first exposure was 6 minutes; eggs so treated without a second exposure gave membranes and some cleavage but no blastulæ. After each interval the eggs kept in sea-water received second exposures of 6, 8, and 10 minutes; those kept in KCN sea-water received 4, 6, 8, and 10 minutes.

Interval between exposures (*hours*)	Percentage of eggs forming blastulæ and optimum durations of second exposure	
	A. Eggs kept in normal sea-water	B. Eggs in M/1000 KCN in sea-water
1	35–40 (8 and 10 min.)	*ca.* 50 (6–8 min.)
3	few (1%) (6–8 min.)	50–65 (4–6 min.)
5	0 (6–8 min.)	25–35 (4–8 min.)
22	(all dead by 22 hrs.)	0

Sperm-fertilized eggs developed normally.

Well-marked response to the second exposure after 5 hours in M/1000 KCN was observed in four other similar experiments and also in three experiments in which the eggs were left for 5 hours in oxygen-free sea-water between exposures. Four experiments with periods of 18 to 22 hours in M/1000 KCN showed almost no response after this interval, although in one experiment some slight effect was apparent after 19 hours.

Sea-water deprived of oxygen (by prolonged boiling, restoration of the original volume with boiled distilled water, and passage of a stream of hydrogen or nitrogen overnight) prolongs similarly the possible summation-interval. For example, on August 16, 1927, eggs kept for 2 1/2 and 5 hours in oxygen-free sea-water, after a first exposure of 5 minutes to .002 M acetic acid, yielded with both intervals 60–70 per cent of blastulæ as the result of a second exposure of 6 to 8 minutes to the same solution. The control eggs kept in normal sea-water showed almost no response to the second exposure. The eggs which received only the first exposure of 5 minutes showed typical partial activation with no blastulæ, while those receiving continuous exposures of 12, 13 and 14 minutes at the same time (30 minutes after removal from the animals) gave 75–90 per cent of blastulæ.

Seventeen experiments of this kind were performed during the summer of 1927 with results similar to those cited. In all cases the interval between the first inadequate and the second effective exposure was prolonged to several hours by the suppression of oxidative metabolism in the manner indicated.

These results are in conformity with the hypothesis that some material essential to the production of the activating substance disappears normally from the egg in the course of the regular oxidative metabolism. Hence the unused reserve of this material is retained within the egg for a longer period, together with the activating substance formed by the first exposure, if oxygen-consumption is prevented.

There is, however, some indication that the reserve of precursor material (activable substance) remaining in the egg after the first exposure is not retained entirely unaltered during the interval, but that it undergoes gradual transformation into activating substance. This is seen in the fact that partially activated eggs show an increase in the degree of their activation (as compared with eggs of the same lot left in normal sea-water), if they are kept undisturbed for some hours in oxygen-free or cyanide-containing sea-water. For example, in the experiment just cited (August 16, 1927) eggs left for two hours in oxygen-free sea-water (after the initial exposure of 5 minutes to .002 M acetic acid), and then transferred to normal sea-water, yielded without any second treatment 10–15 per cent of blastulæ, while after remaining for 5 hours in oxygen-free sea-water more than 50 per cent formed blastulæ. In the latter case (5 hours without oxygen) a second exposure of two minutes proved almost as effective as one of 6 minutes, both yielding 65–70 per cent of blastulæ. Such cases show that a partial activation may be completed in many eggs by simple exposure of one to several hours to cyanide-containing sea-water. This effect is similar to Loeb's completion of activation in sea-urchin eggs, after membrane-formation by acid, by after-treatment with cyanide-containing sea-water (Loeb, 1913). In starfish eggs a well-marked shortening of the optimal duration of the second acid treatment was a constant effect of one or more hours exposure to oxygen-free or cyanide-containing sea-water. Apparently prolonged suppression of oxygen-consumption in the partially activated eggs has a physiological effect similar to that of treatment with acid or high temperature.

It seems probable that the production of acid within the egg under the conditions of anaërobic metabolism is responsible for the increase of activation shown in such cases. This supposition is consistent with the fact that previous exposure of normal untreated starfish eggs to oxygen-free or cyanide-containing sea-water also very definitely increases the susceptibility to activation by either heat or acid, as will be shown in the next section. I have, however, never succeeded in activating such eggs, even to the extent of membrane-formation, by this treatment alone.[5] The acid formed under these conditions may not reach the critical level of concentration required for activation, or it may be ineffective for some independent reason.

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[5] E. P. Lyon obtained parthenogenetic development in the *Strongylocentrotus* egg at Naples by prolonged exposure to solutions of KCN (M/100 to M/1000) in sea-water (*cf.*, *Am. Jour. Physiol.*, 1903, Vol. 9, p. 308).

2. *Activation after Previous Exposure of Normal Eggs to Cyanide and Lack of Oxygen*

In the experiments just described partially activated eggs after remaining some hours in oxygen-free or cyanide-containing sea-water showed a tendency to complete their activation spontaneously; *i.e.*, the second effective exposure to acid was unexpectedly brief, and many eggs formed blastulæ without any second exposure. Similarly, normal unactivated eggs kept under the same oxidation-suppressing conditions show in course of time an increasing susceptibility to activation by either heat or acid. Suppression of oxygen-consumption has thus a double effect on the eggs: (1) prolongation of the time during which they remained activable, and (2) increase of activability, as shown by a decrease in the effective durations of exposure to the activating agent.

Cyanide-containing Sea-water.—A typical series in which the eggs were exposed to KCN-containing sea-water for 2 1/2 hours before the activating treatment is the following:

July 3, 1929. Eggs from several starfish were placed, 35 minutes after removal, in sea-water containing M/1000 KCN. Two hours, 35 minutes later, a part (*B*) was washed in several changes of normal sea-water, left in sea-water for 26 minutes and then placed in M/260 butyric acid solution in sea-water. A second part (*C*) after 2 hours, 32 minutes in the KCN sea-water was transferred directly to KCN sea-water containing also M/260 butyric acid.[6] The essential difference between *B* and *C* is that in *B* the activating treatment occurred after the eggs were washed free of cyanide, while in *C* the entire period preceding and during activation was spent in the presence of cyanide. At two-minute intervals eggs were transferred from the butyric acid solutions to normal sea-water; later the proportions developing to blastulæ were determined. The control (*A*) consisted of eggs exposed to M/260 butyric acid in sea-water, beginning 40 minutes after removal from the animals.

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[6] In estimating the rate of activation in solutions of fatty acid containing KCN, allowance must be made for the neutralizing action of this salt, which is very nearly the same as that of $NaHCO_3$. In general the rate of activation exhibited by starfish eggs placed directly from sea-water in solutions of fatty acid containing also KCN is closely similar to that of eggs exposed to solutions of the same non-neutralized acid strength without KCN. This was shown experimentally as follows: Eggs exposed to balanced $NaCl$-$CaCl_2$ solution containing both acetic acid and KCN in equal concentrations, .002 M, showed no membrane formation or other signs of activation with exposures from 5 to 18 minutes. The control experiment with .002 M acetic acid alone showed optimal activation at 10 minutes, with 75–80 per cent of eggs forming blastulæ. The same $NaCl$-$CaCl_2$ solution containing .002 M acetic acid plus .001 M KCN gave a rate of activation similar to that of .001 M acetic acid without KCN. The solution used above, sea-water containing M/260 butyric acid *plus* M/1000 KCN, corresponds closely in its activating effect on normal eggs to a solution of .003 M butyric acid in sea-water (*i.e.*, M/260, *ca.* .0038–.001 M).

Table III gives the approximate percentages of eggs forming blastulæ with the different exposures.

TABLE III

Duration of exposure (20° C.) (*minutes*)	Percentage of eggs forming blastulæ		
	A. (Control without KCN exposed to normal sea-water containing M/260 butyric acid)	B. (KCN 2½ hrs. exposed to normal sea-water containing M/260 butyric acid)	C. (KCN 2½ hrs. exposed to sea-water containing M/260 butyric acid *plus* M/1000 KCN)
2	0	40–50	no observation
4	1	30–40	60–70
6	15–20	30–40	60–70
8	30–40	60–70	50
10	*ca.* 90	70–80	20–30
12	80–90	70–80	*ca.* 10
14	70–80	30–35	*ca.* 10
16	20–30	*ca.* 10	1

Nearly half the eggs in Part *B* formed blastulæ with exposures as brief as two minutes; also a large proportion continued to develop favorably with longer exposures up to 12 minutes. The effect is even more striking in Part *C*. Here the effective (non-neutralized) concentration of acid in normal sea-water (*ca.* .003 M) typically requires 20 minutes or more for complete activation; yet after the preliminary treatment with KCN, two-thirds of the eggs formed blastulæ with exposures of 4 to 6 minutes.

Two other series on July 15 and 16, with preliminary exposures of 3 and 5 hours (respectively) to KCN sea-water gave closely similar results. In the series of July 15 eggs exposed to M/260 butyric acid in sea-water, after 3 hours in KCN sea-water, for periods of one to 6 minutes, gave 60–80 per cent blastulæ. With the cyanide-containing butyric solution one minute was ineffective, but exposures of 2 to 6 minutes yielded 50 per cent or more blastulæ. The results of July 16, with 5 hours previous treatment with KCN, were similar; butyric acid in normal sea-water showed optimal activation at 2 minutes with 70–80 per cent blastulæ; the same solution in KCN-containing sea-water was ineffective at 2 minutes, but with exposures of 4 to 14 minutes gave 50 per cent or more blastulæ. On July 17 and 18 similar series with 5 and 3 hours in KCN-sea-water showed the same effect with somewhat fewer blastulæ.

The shortening of the effective exposures under the influence of KCN is appreciable although slight after 15 or 20 minutes and increases with time. Table IV gives the results of a typical series in which the preliminary exposure to KCN was varied from 15 to 65 minutes. The optimum exposure for the control eggs unexposed to KCN was 10 minutes (21° C.), with more than 90 per cent forming blastulæ.

Table IV

Experiment of June 20, 1930. Eggs were exposed during the prematurational period to M/260 butyric acid in normal sea-water at 21° C. after washing free of cyanide.

Previous Exposure to M/1000 KCN (*minutes*)	Times of Exposure to Butyric Acid (*minutes*) and Percentages of Eggs Forming Blastulæ								
	2	3	4	5	6	7	8	9	10
None (control)	0	0	0	2–3	2–3	5–10	*ca.* 50	65–75	90
15	0	0	0	2–3	2–3	60–70	60–70	75–85	80–90
26	0	*ca.* 1	*ca.* 1	*ca.* 1	25–35	50–60	70–80	70–80	*ca.* 50
45	1	*ca.* 5	10–20	40–50	60–70	65–75	50–60		
65	*ca.* 1	*ca.* 5	10–15	20–25	35–45	55–65	25–35		

Three other similar series carried out between June 17 and June 23, 1930, with exposures to KCN ranging from 12 minutes to 5 hours, gave the same general result. In general, the degree of shortening increases with duration of exposure up to a maximum of 2 to 3 hours. After 5 hours there is usually a decline of responsiveness, and after 20 to 24 hours no blastulæ were obtained, although there was some partial activation.

Oxygen-free sea-water.—Experiments with eggs remaining for 2 to 5 hours in oxygen-free sea-water before activation with fatty acid gave a similar result.[7] Eight successive series between July 23 and August 3, 1929 showed uniformly a striking decrease in the activating exposures; of such eggs a large proportion and in some cases nearly all formed blastulæ after exposures of one to 2 minutes to M/260 butyric acid. Table V gives a summary of the first four series. The eggs, after exposure to the oxygen-free sea-water (in flasks through which a slow stream of hydrogen was kept flowing) for the periods named, were treated for periods ranging from one to 16 minutes with M/260 butyric acid made up in both normal and oxygen-free sea-water. The controls, as before, consisted of eggs exposed during the prematurational period (usually 30 to 45 minutes after removal) to M/260 butyric acid,[8] again both with and without oxygen. These showed optima varying between 6 and 12 minutes (most frequently at 8 minutes) with 70 to 85 per cent of eggs forming blastulæ. The temperatures of the sea-water and butyric acid solutions were 21° to 22.5° C.

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[7] In general the physiological effects of exposing starfish eggs to cyanide-containing and to oxygen-free sea-water are strikingly similar. One constant effect following exposure for one to several hours to either medium is to render the eggs glutinous or sticky, so that they cohere in masses, a result (apparently) of the secretion of some water-swelling or glutinous material.

TABLE V

Experiment and date (1929)	Control (Effective exposures and percentages of eggs forming blastulæ)		Period in O_2-free sea-water before activation		Exposures to M/260 butyric acid and percentages of resulting blastulæ			
					(a) O_2-containing acid solution		(b) O_2-free acid solution	
	min.	%	*hrs.*	*min.*	*min.*	%	*min.*	%
1. July 23	6	(35–40)	2	15	2	(*ca.* 50)	2	(*ca.* 50)
	8	(70–80)			4	(35–45)	4	(50–60)
	10	(50–60)			6, 8	(25–35)	6	(35–40)
					10	(20–35)	8, 10	(25–35)
							12	(20–25)
2. July 24	4	(25–35)	5		1	(40–50)	1	(35–40)
	6	(80–90)			2, 3	(20–30)	2	(30–35)
	8	(50)			4	(5–10)	3	(15–20)
	10	(5)			6	(0)	4	(*ca.* 1)
3. July 25	6	(40–50)	4	25	1, 2	(80–90)	1	(75–85)
	8	(75–80)			3, 4	(75–85)	2, 3	(80–90)
	10	(*ca.* 90)			5	(70–80)	4, 5	(70–80)
	12	(50–60)			6, 7	(*ca.* 60)	6, 7	(65–75)
					8	(50)	8	(55–60)
4. July 26	8	(10–15)	3	40	2, 4	(70–80)	2, 4	(70–80)
	10	(40–50)			6, 8	(60–70)	6	(60–65)
	12, 14	(70–80)			10	(*ca.* 50)	8	(*ca.* 50)
					12, 14	(25–35)	10	(30–40)
							12	(*ca.* 5)

In three other series (August 1, 2 and 3, 1929) eggs kept previously for equal times in both KCN-containing and oxygen-free sea-water (respectively 2 1/4, 3 and 4 1/2 hours) were exposed to M/260 butyric acid in sea-water. In all cases exposures of 1, 2 and 3 minutes gave numerous blastulæ (60 to 80 per cent in five of the six experiments); exposures of 4, 5 and 6 minutes were less effective, and with 8 minutes almost no blastulæ were formed. The control eggs gave no blastulæ with the two-minute exposure, few with 4 minutes and a maximum (60–80 per cent) with 6 to 8 minutes.

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[8] Two cc. M/10 butyric acid *plus* 50 cc. sea-water. Oxygen-free sea-water was used in mixing solution (*b*) and hydrogen was bubbled through the mixture for some hours.

One uniform feature of these experiments was that the eggs, after prolonged suppression of oxidative metabolism, not only gave complete activation with brief exposures to acid, but also showed a less sharply defined optimal period of exposure; *e.g.*, in the experiments of July 25th and 26th exposures ranging from one to 8 minutes were almost equally effective in producing blastulæ (65 to 85 per cent). This behavior differs strikingly from that of eggs activated in normal sea-water soon after removal; in this case the curve relating percentages of blastulæ to durations of exposure rises rapidly to a maximum—usually at 8 to 10 minutes at this temperature (21°–22° C.) and concentration of acid—and declines equally rapidly, appearing nearly symmetrical.[9] Under the anaërobic conditions just described, over-exposure seems less deleterious; the optimum is reached early, and with further prolongation of the activating treatment there is a more gradual decline of effect; *i.e.*, over-activation is caused less readily, an indication (possibly) of a partial depletion of activable or precursor material during the interval in oxygen-free sea-water. A similar prolongation of the optimum is seen in eggs remaining for some hours in KCN-sea-water previous to activation.

Controls of sperm-fertilized eggs were also kept during these experiments: at the end of the period in oxygen-free or KCN-containing sea-water eggs were transferred to normal sea-water, washed by changing the latter, and immediately fertilized by sperm. In general, responsiveness to sperm fertilization was found to run closely parallel with activability by artificial agents; *i.e.*, normal fertilizability is also prolonged for several hours by exposure to anaërobic conditions. Typically, sperm-fertilization is complete and uniform only during the prematurational period and falls off rapidly with the separation of the polar bodies (Lillie, 1908). Both kinds of prolongation of responsiveness are to be regarded as expressions of the same kind of change in the egg; in terms of the foregoing hypothesis, the precursor material, which is transformed into activating substance under the influence of both acid and sperm, is retarded in its breakdown or removal by the suppression of oxidative metabolism.

3. *Influence of O_2-lack and KCN on Activation by Heat*

The effective exposures to activating temperatures (*e.g.*, 32°) are similarly shortened by previous exposure to cyanide or lack of oxygen.

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[9] *Cf.* the curves in my article on activation by acids in *Jour. Gen. Physiol.*, 1926, Vol. 8, p. 339.

In general, however, I have found that in the case of heat activation the decline in responsiveness during such exposure is more rapid than with acid activation. This may indicate that the material which gives rise to the activating acid (presumably carbohydrate) undergoes comparatively rapid depletion with time. In some cases, the eggs have preserved good responsiveness to heat for as long as 3 hours in KCN-containing sea-water, although usually the response is greatly diminished by this time. Apart from this difference the general phenomena are closely similar.

The procedure was the same as in my earlier experiments on heat activation (Lillie, 1908, 1915). The eggs, together with a small quantity of sea-water, were placed in a beaker (or small flask) to which 100–200 cc. of sea-water at the required temperature (32°) was added. The temperature was kept constant by a water bath. At intervals eggs were transferred to a series of dishes containing normal sea-water.

The following experiment (August 6, 1929) is cited as an illustration. One portion of starfish eggs (*A*) was placed, one half hour after removal from the animals, in M/1000 KCN in sea-water; another portion (*B*) in oxygen-free sea-water; in this case one to 2 cc. of a dense egg suspension was added to 100 cc. of oxygen-free sea-water contained in a flask through which hydrogen had flowed overnight, and a slow flow of H_2 was continued. After 3 hours eggs from both lots were treated with normal sea-water at 32° for the times indicated. It had previously been found that eggs left in normal sea-water for 2–3 hours, and then warmed, show only partial response and never form blastulæ (Lillie, 1908).

TABLE VI

Treatment	Percentage of eggs forming blastulæ after exposure to 32° C. for the times indicated (*minutes*)							
	1	2	3	4	5	6	7	8
1. Control: warm sea-water, 30 min. after removal...........	0	1	2–4	25–35	75–85	75–85	60–70	25–35
2. (*A*) In KCN-sea-water 3 hrs., 10 min. before warming.....	10–15	25–35	*ca.* 50	25–35	20–30	1–2	0	0
3. (*B*) In O_2-free sea-water 3 hrs., 10 min. before warming.....	*ca.* 1	*ca.* 5	20–25	30–35	5–10	1–2	0	0

The preservation of responsiveness and the shortening of the effective exposures are both well shown in this series. In six other similar experiments during the same season, with exposures of one hour or more to KCN, the effective exposures were similarly decreased, but fewer eggs formed blastulæ. With a briefer previous stay in KCN-containing-sea-water (19 to 24 minutes in five experiments between June 25 and July 1, 1930), the decrease was less marked but still definite. The actual exposure to heat may be made either in normal or in KCN-containing sea-water with the same result. Even without a previous stay in KCN-containing-sea-water, eggs warmed in KCN-containing or oxygen-free sea-water show a distinctly briefer optimal exposure than eggs of the same lot warmed in normal sea-water. Table VII gives a summary of experiments illustrating these effects.

TABLE VII

Procedure	Percentages of eggs forming blastulæ after exposure to 32° for the times indicated (*minutes*)								
	1	2	3	4	5	6	7	8	9
1. June 25, 1930.									
(*a*) Control: normal sea-water at 32°	0 (no membranes)	0 (few membranes)	0 (all form membranes)	0	10–15	10–15	20–25	30–35	*ca.* 10
(*b*) No previous exposure to KCN; KCN-containing sea-water at 32° at same time as control	0 (membranes in nearly all eggs	0	0	10–20	20–30	35–40	5–10	0	
2. June 28, 1930.									
(*a*) Control: normal sea-water at 32°	0 (no membranes)	0 (50% membranes)	0 (all membranes)	*ca.* 1	25–35	60–65	*ca.* 60	10–20	
(*b*) Previous exposure to KCN sea-water for 17 minutes; then direct to KCN-sea-water at 32°	0 (all form membranes)	0	5–10	15–25	60–70	20–30	1	0	

Lack of oxygen has the same effect as KCN upon the rate of heat activation. One uniform and readily observed effect is the shortening of the exposures required for membrane-formation. Typically eggs exposed to normal sea-water at 32° for one minute do not form membranes or show any other signs of activation; exposures of 2 1/2 to 3 minutes are required to form membranes in a majority of eggs. But if eggs are warmed in KCN-containing or O_2-free sea-water (especially after previous exposure to either medium) for one minute, all (or nearly all) form membranes (*cf.* Table VII). Restoration of normal oxygen-consumption reverses this sensitivity,—*i.e.*, eggs exposed as above to oxygen-free or cyanide-containing sea-water and then returned for a few minutes to normal sea-water are found to require 2 or 3 minutes at 32° for membrane-formation, like previously untreated eggs.

Membrane-formation is an index of partial activation and is a regular effect of brief exposure to either heat or acid, as well as to other parthenogenetic agents such as solutions of cytolytic compounds and pure isotonic alkali salt solutions. Complete activation, however, is obtained only with heat and acid,[10] but with these agents the exposures required for the formation of blastulæ are several times longer than those required for membrane-formation alone. In confirmation of the hypothesis that heat acts indirectly through the intra-cellular production of acid, the following experiment is cited. Eggs were exposed to neutral, acid and alkaline artificial unbuffered sea-water (van't Hoff's solution) [11] at 34° as indicated in Table VIII.

TABLE VIII

Solution in which eggs were warmed (34°)	Times of exposure (*minutes*) and percentages of resulting blastulæ								
	1	1½	2	2½	3	3½	4	4½	5
1. Neutral van't Hoff	0 (*ca.* 50% membranes)	2–3	20–30	65–75	50–60	*ca.* 50	15–20	10–15	1
2. van't Hoff *plus* N/1000 NaOH	0 (40–50% membranes)	*ca.* 5	30–40	*ca.* 90	*ca.* 90	75–85	20–30	1–2	0
3. van't Hoff *plus* N/1000 HCl	25–30 (*ca.* 100% membranes)	55–65	75–85	20–30	1	0	0	0	0

Neutral or moderately alkaline balanced isotonic salt solution at 34° has the same action as sea-water at this temperature, while acidulation to this degree nearly doubles the rate of activation. This is apparently a summation effect resulting from a partial penetration of the external acid to the site of activation. The increased rate of activation observed in KCN-sea-water may similarly be regarded as a summation effect, due to the increased intracellular formation of acid under the conditions of asphyxiation. With regard to the reversal of hypersensitivity just described, it may be assumed that readmission of oxygen restores the egg to its original condition through the oxidative removal of the surplus of acid, as in the case of the lactic acid formed in muscle cells under asphyxia.

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[10] *I.e.*, apart from hypertonic sea-water (*cf.* next section), which apparently acts indirectly through its influence on metabolism. In numerous experiments with cytolytic compounds (*e.g.*, ether and chloroform), pure Na-salt solutions, ultra-violet radiation and the electric current, I have never obtained more than partial activation, whatever the duration of exposure.

[11] Made by mixing M/2 solutions of the following salts in the proportions by volume: 100 NaCl, 7.8 $MgCl_2$, 3.8 $MgSO_4$, 2.2 KCl, 2.0 $CaCl_2$.

Experiments in which the eggs were placed for 19–20 minutes in sea-water saturated with pure oxygen from an oxygen-cylinder and then warmed to 32° in the same medium showed no significant difference from the control. (Results of three series: July 15, 16 and 18, 1930.) Apparently an increase of oxygen tension to 5 times the normal does not affect the rate of heat activation.

4. *Influence of O_2-lack and KCN on Activation by Hypertonic Sea-Water*

The case of hypertonic sea-water is peculiar. This agent, acting alone, is not so effective with starfish eggs as heat or fatty acid, and its rate of action is much slower. Nor is it so effective with these eggs as with *Arbacia* eggs. It is unusual to obtain 50 per cent of free-swimming blastulæ with hypertonic sea-water alone, although in a few experiments the proportion has reached 70 per cent or even higher. The most favorable procedure is to expose the eggs during the prematurational period to a mixture of 100 volumes sea-water *plus* 20 volumes 2.5 M NaCl for 1 1/2 to 2 hours (at 20–22°). Exposures during the post-maturational period are much less effective. In experiments with a series of hypertonic mixtures, consisting of 100 volumes sea-water *plus* (respectively) 10, 15, 20, 25 and 30 volumes 2.5 M NaCl, membrane-formation and blastulæ were obtained with all but the first and last. The effective osmotic gradient thus has a well-defined range; and within this range the optimal exposures show in general a decreasing duration with increasing concentration, as in Loeb's experiments with *Strongylocentrotus*.[12] The best results have been obtained with the 100 + 15 and 100 + 20 mixtures, corresponding to an increase in osmolar concentration of 50 to 60 per cent. The second of these solutions was used in most experiments.

Besides acting as a single parthenogenetic agent, hypertonic sea-water may also be used to supplement and complete a partial activation by heat or fatty acid (See Lillie, 1915, pp. 284 *seq.*).

A characteristic peculiarity in the mode of action of this agent is that the physiological change which it produces in the egg during the period of exposure is closely associated with the consumption of oxygen. In this respect the action of hypertonic sea-water offers a contrast to that of fatty acid and heat, both of which agents act independently of immediate oxygen consumption, besides being more uniform, rapid and complete in their activating effect.

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[12] J. Loeb: Artificial Parthenogenesis and Fertilization, Chapter XI.

Loeb showed many years ago that oxygen-free or KCN-containing hypertonic sea-water was ineffective as an after-treatment in sea-urchin eggs which had received the initial membrane-forming treatment.[13] In starfish eggs similar conditions are found, except that in order to deprive the hypertonic sea-water completely of its activating influence, it is necessary to expose the eggs previously for some time to cyanide or lack of oxygen. Eggs transferred from normal sea-water directly to oxygen-free or KCN-containing hypertonic sea-water always show some partial activation (*i.e.*, membrane-formation and occasionally a few blastulæ); while if they are kept first in oxygen-free (or KCN-containing) isotonic sea-water for 2 to 3 hours and then exposed to the oxygen-free (or KCN) hypertonic sea-water for the usual time (1 1/2 to 2 hours) the great majority show no signs of activation. If such eggs are then returned to sea-water and fertilized with sperm, a large proportion develop to larval stages, showing that they have remained essentially unchanged in their properties.

These conditions are illustrated by the experiments described in Tables IX and X.

TABLE IX

June 26, 1929. The eggs were placed, 40 minutes after removal, in (*A*) hypertonic sea-water of the composition 100 volumes sea-water *plus* 20 volumes 2.5M NaCl, and (*B*) in the same solution *plus* M/1000 KCN. Temperature 21° C. At intervals of 1½, 1¾ and 2 hours portions were transferred to normal sea-water.

Duration of exposure (*hours*)	Percentage of mature eggs forming blastulæ	
	A. Hypertonic sea-water	B. Hypertonic sea-water *plus* M/1000 KCN
1½	70–80	0
1¾	60–70	1–2
2	60–70	*ca.* 5

The eggs in this experiment were exceptionally responsive to hypertonic sea-water; it is in fact unusual to obtain any blastulæ after treatment with KCN-containing hypertonic sea-water. In a similar experiment on June 27 the eggs exposed to cyanide-free hypertonic sea-water for 1 1/2 hours at 23° formed 25 per cent of blastulæ, while with KCN present, although all formed membranes and many cleaved irregularly or fragmented, no blastulæ were obtained. Other experiments gave similar results.

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[13] *Loc. cit.*, Chapter XI.

The effect of placing in oxygen-free or KCN-containing sea-water for some time previous to the hypertonic treatment is shown in the following experiments (August 1, 1929).

TABLE X

Procedure	Result
A. Eggs exposed to hypertonic sea-water (100 + 20) alone, beginning 30 min. after removal, for 1½ and 1¾ hours (23°)	Activation in all; 25–35 per cent blastulæ
B. Eggs in O_2-free sea-water for 2 hours, beginning 30 min. after removal; thence to O_2-free hypertonic sea-water for 1½ and 1¾ hours	Great majority show no activation (no membranes); partial activation in a few
C. Eggs in sea-water + M/1000 KCN for 2 hours, beginning 35 minutes after removal; thence to KCN-containing (M/1000) hypertonic sea-water for 1½ and 1¾ hours	Similar to Experiment B. No activation except in small minority

A similar result was obtained in eight other experiments of the same kind, five with KCN-containing and three with oxygen-free hypertonic sea-water. It is evident that when the oxidative metabolism of the egg is suppressed, the abstraction of water by hypertonic media has little or no activating effect. The period of asphyxiation does not in itself deprive the eggs of responsiveness to hypertonic treatment, provided the oxygen is later restored. Eggs returned after 2 to 3 hours in KCN-containing sea-water to normal sea-water, washed thoroughly in sea-water and then exposed to hypertonic treatment show typical activation with production of blastulæ. As already shown, such eggs also retain their responsiveness to activation by heat and acid and to sperm-fertilization.

THEORETICAL

Assuming that the activation process consists in a regular sequence of interconnected physical and chemical processes, leading to some definite modification of the egg-system, we have to inquire how such a sequence can be initiated by a temporary slight rise of acidity. In general the experimental facts suggest that the first step in the chemical sequence of activation is a transformation (*e.g.*, hydrolysis) of some compound present in the surface layer or cortical zone of the egg, this change leading directly or indirectly to the formation of some specific metabolic product ("activating substance"), the accumulation of which renders the egg capable of automatic development. Just how such a substance may be regarded as producing its effect is uncertain; it might contribute to special cell-structure, or alter already existing structure (*e.g.*, increase permeability), or it might be a catalyzer, or form some necessary link in the chain of structure-forming reactions. On the whole the last supposition seems the most likely one. Examples of the control of morphogenetic processes by special metabolic products are well known (*cf.*, the case of hormones); and they show at least that the hypothesis of an activating substance is consistent with the general facts of developmental physiology.

It seems unlikely that the primary or releasing reaction (or reactions) of activation can be widely different from those met with in other types of reactive cell. In general the basic chemical processes of protoplasm (as well as its basic chemical composition) show a well-defined uniformity throughout the range of living organisms. Thus we find that the carbohydrate metabolism of yeast cells resembles closely that of muscle cells, with certain divergences in detail; the utilization of oxygen by living cells depends on factors of a kind universally present in protoplasm (*cf.*, the heme and sulphydryl compounds); the course and end-products of protein metabolism are similar in their general character. Such considerations lead us to expect that the key reactions of activation may be simple and of a type already known.

The prompt arrest of the activation reaction when eggs are returned from acid to normal sea-water, and its equally prompt renewal on re-exposure to acid, are facts suggesting that the rise of acidity within the egg acts by releasing from combination some compound which is necessary for the specific reaction of activation, and that this releasing reaction is reversed on return to or toward neutrality, the compound re-entering combination and becoming again unavailable. The releasing reaction itself has apparently the character of a hydrolysis, and the dependent specific reaction which forms the activating substance is to be regarded as keeping pace with this hydrolysis.

Certain readily hydrolyzable compounds widely distributed in cells show a behavior of the general kind required by such a hypothesis. Of chief interest are the phosphate compounds or esters, especially the guanidin phosphates (phosphagens), which appear to be of special importance in the metabolic reactions associated with excitation in muscle. The enzymatic hydrolysis of these compounds in muscle extracts is influenced by variations of acidity in a manner closely paralleling the characteristic variation in the rate of activation of eggs with concentration of acid. The creatin phosphate of vertebrate muscle extracts splits into creatin and phosphoric acid at a slightly acid reaction (pH = 6.4), and is resynthesized at slight alkalinity (pH = 8.5–9); the analogous arginin phosphate of invertebrates shows similar properties, but has a greater tendency to resynthesis at slightly alkaline or even neutral reaction.[14] The total phosphate content of starfish eggs is very similar to that of muscle cells (Page, 1927), and it is possible that in the activation and other reactions of these cells phosphate esters play a rôle of the same kind as in the analogous reactions of muscle and presumably other cells. Meyerhof has suggested that a special biological rôle of the guanidin compounds may consist in the binding and splitting off of phosphate groups (Meyerhof and Lohmann, 1928), a view in accordance with the general importance of phosphates in cell metabolism. The hypothesis that the splitting of phosphagen or some analogous compound is the primary chemical event in activation requires, however, further experimental testing from both the physiological and the biochemical sides.[15]

With regard to the activation by hypertonic sea-water, it is to be noted that abstraction of water from the egg means increase in concentration of egg-constituents, and it is possible that locally (*e.g.*, in the cortical region) this increase may be considerable. Conditions favorable to the synthesis of complex compounds would thus arise. The general theory of the dehydrolytic synthesis of complex molecules through the combination of a number of smaller molecules has recently been reviewed by Wasteneys and Borsook (1930, 1931) in relation to their work on the enzymatic synthesis of plasteins from the products of peptic hydrolysis. The authors show both theoretically and experimentally that increase in the concentration of such a digest may change the prevailing direction of the reversible reaction from a hydrolysis to a synthesis; and it is reasonable to assume that conditions of the same general kind exist in living cells. In the case of the starfish egg the inference would be that hypertonic sea-water acts by promoting the synthesis of some compound (*e.g.*, protein) which either is, or gives rise to, the activating substance.

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[14] Meyerhof and Lohmann, *Biochem. Zeitschr.*, 196, 49, 1928. It might be objected that such reactions are too slow to account for the rapid course of the activation reaction in starfish eggs immersed in acid sea-water, and its rapid arrest on return to normal sea-water; but it is well-known that many enzymatic reactions which are slow *in vitro* proceed rapidly in the living cell.

[15] *E.g.*, it would be important to determine if the free phosphate is increased in the egg during activation, as in the stimulation of muscle and nerve.

It is significant that the formation of the activating substance appears to be promoted by two quite different external changes of condition, hypertonicity and moderate acidity. As already described, hypertonic activation proceeds relatively slowly as compared with acid activation and is dependent on oxygen consumption. The essential problem is why dehydration combined with oxidation should lead to the production of the same substance as slight local increase of acidity. Experimentally we find that the two parthenogenetic procedures may act additively or supplement each other; according to the present hypothesis, however, they influence metabolism in different ways, the one promoting synthesis of a complex compound, the other releasing hydrolytically some relatively simple compound which enters further combination. The fact that both procedures have the same final physiological effect suggests that the activating substance is formed by the combination of the two products. In such a case, according to the mass action rule, increase in the concentration of the one product would offset deficiency in the other; *i.e.*, activation would result from a sufficient increase in either compound, provided some of the other were available. This would explain why either acid or hypertonic sea-water acting alone may cause complete activation, since both compounds may be assumed to be present in low concentration in the resting egg. Whether the additive relations between the two parthenogenetic procedures conform to such a hypothesis in detail can be determined only by further experiment.

SUMMARY

1. Suppression of oxygen-consumption in freshly removed unfertilized starfish eggs (by exposure to cyanide-containing or oxygen-free sea-water) prolongs by several hours the period during which they remain responsive to artificial activation by heat, acid, or hypertonic sea-water (as well as to sperm fertilization). The possible interval between a first partial and a second completing activation may be similarly prolonged.

2. During the exposure of the eggs to these asphyxiating conditions their susceptibility to activation by heat or acid, as indicated by the effective durations of exposure, undergoes a progressive increase.

3. Eggs kept for some hours in cyanide-containing or oxygen-free sea-water and then exposed to acid or heat while immersed in these media show normal activation. On the other hand, a similar suppression of oxygen consumption prevents activation by hypertonic sea-water.

4. It is suggested that the activation by hypertonic sea-water de-

pends on the increased intracellular production, by dehydrolytic synthesis, of some complex specific compound (*e.g.*, protein); while in the anaërobic activation by acid (or heat) the critical change is a hydrolysis (*e.g.*, of a phosphagen compound), yielding a product which combines with the complex compound to form the specific activating substance. The accumulation of this substance to a certain definite level determines activation. Two metabolic processes, respectively aërobic and anaërobic, would thus coöperate in activation. The fact that either hypertonic sea-water or acid (or heat), acting alone, can produce the same physiological end-effect, complete activation, is shown to be consistent with this hypothesis.

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MODIFICATION OF THE RATE OF OXYGEN CONSUMPTION BY CHANGES IN OXYGEN CONCENTRATION IN SOLUTIONS OF DIFFERENT OSMOTIC PRESSURE

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Introduction

It has not been possible to derive a general rule describing the relation between rate of oxidations in organisms and the oxygen tension of the environment. Pflüger (1872), reasoning from observations on the oxygen-absorbing power of the blood and the partial pressure of oxygen in the alveoli of the lungs in the dog, concluded that the rate of oxygen absorption of the tissues is independent of the oxygen tension over a wide range. Thunberg (1905), testing the oxygen absorption of *Lumbricus*, *Limax*, and *Tenebrio* larvæ in different oxygen tensions, found that the rate varies with the oxygen tension. Thunberg assumed that the oxygen tension within the cells is less than that of the milieu, but not zero, and concluded that his results were in accord with the fact that the velocity of a reaction depends on the number of molecules reacting; hence the velocity of oxidations in the organism varies with the oxygen concentration of the environment until a maximum is reached. Henze (1910), from his experiments with pelagic forms, concluded that in organisms with thin and delicate tissues the oxygen tension of the cells is that of the surrounding water and always in excess of requirements; consequently in such organisms the rate of oxygen utilization is independent of the oxygen tension of the water.

Krogh (1916) amplified Henze's conclusion and states that whether or not the rate of oxygen consumption is independent of the oxygen concentration of the environment depends on two conditions: In animals with well developed blood streams and respiratory systems, and in animals with a low percentage of dry substance, the oxygen tension does not affect the rate of oxygen utilization over a wide range. The assumption is necessary in these cases that the oxygen tension in the tissues is positive. On the other hand, if the organism has a high proportion of dry substance and "imperfect" respiratory and circulatory systems, the rate of oxygen utilization is highly dependent on the oxygen tension of the environment until the oxygen tension is above the normal. It is assumed that the oxygen tension of the tissues of such forms approaches zero.

Hyman (1929) suggests that, in certain cases at least, the body surface acts as a regulatory mechanism; this appears to act according to internal requirements and hence in the presence of excess oxygen the oxidative rate remains constant. Shoup (1929) holds that the size of the organism is important. In general, according to Shoup, microscopic forms are small enough to allow complete diffusion of the dissolved gases. In other words, they are independent of the oxygen concentration, within limits, because oxygen is always present in excess within the system. Shoup also adopts the view expressed by Krogh (1916) that in large organisms the rate of respiratory exchange depends on oxygen tension and the adequacy of the respiratory and circulatory systems.

Thus far it is not possible to explain the experimental evidence at hand by any or all of these theories. According to Krogh, the water content of the organism is an important characteristic in determining its response to changes in the oxygen tension of the environment. Thus an organism with relatively low water content and what Krogh calls "imperfect" systems of respiration and circulation might be expected to consume oxygen in direct dependence on the oxygen concentration of the environment. In the case of *Planaria dorotocephala* certainly the mechanisms of respiration and circulation are, so far as known, simple and rudimentary; yet Hyman (1929) found that this form has a constant rate of oxygen utilization over a range of environmental oxygen tension between 3 cc. and 9 cc. per liter. If Krogh's suggestion has a basis in fact, one would expect that the water content of this animal is relatively high.

Planaria afford suitable material for a further study of the relation between water content and the degree of dependence of rate of oxygen consumption on the oxygen tension of the milieu. More specifically, the problem at hand is: What is the mechanism that renders the rate of oxygen utilization of this animal indifferent to changes in the oxygen tension of the surrounding medium? Krogh's suggestion indicates the point of attack, namely, the relation between water content and the effect of changes in oxygen tension on the rate of oxygen utilization with changes in free water content of the organism.

METHODS [1]

Investigation of this problem resolved itself into (*a*) finding the total free water content of *Planaria dorotocephala;* (*b*) increasing and decreasing the water content by means of hypo- and hypertonic solutions; (*c*) determining the rate of oxygen consumption of the animals in different concentrations of oxygen in natural water, and in hypo- and hypertonic solutions.

The free water content was determined by weighing the animals after removal from water, then drying over sulphuric acid in a desiccator, and weighing repeatedly until no further decrease in weight occurred. The first weighing was accomplished as follows: The animals were poured in water into a properly folded hard-surfaced filter paper, the water drained off, and the paper drawn across a plate of glass until it adhered firmly. The animals were then picked up with a thin spatula and placed in a weighing tube, the tube closed, and the weighing completed as rapidly as possible. The proceeding was timed so that the exposure of the animals to air was approximately the same in different experiments. Weighing the animals while wet introduces the error of the weight of water adherent to their surfaces. Considerable success was realized in standardizing this error, for during exposure to sulphuric acid the animals lost very nearly the same percentage of total weight in all experiments; which indicates that the original proportion of adherent water must have been much the same in each first weighing. The total loss of weight as shown by the data must be discounted by the weight of adherent water at the first weighing. The actual water content of the animals is therefore somewhat less than the data indicate.

The degree of dryness attained by desiccation over sulphuric acid depends on the relative affinities of the acid and of the animal tissues for water. The method was used by Davenport (1899) in his analysis of the changes in water content of the frog embryo during development. For the present work the method is more satisfactory than heating to dryness, for only free water is withdrawn from the organism. Heating to dryness involves loss of bound water as well as other substances. It is not assumed, however, that drying over sulphuric acid actually removes all the free water from the system, for the resistance of aquatic organisms to desiccation is common. However, the proportion of water remaining after the treatment must be very small; the residue is a brown mass that on pressure breaks up into a coarse granular powder.

[1] The experimental work on which this article is based was done in the Osborn Zoological Laboratories, Yale University. Acknowledgment is here made for the facilities and assistance provided.

The hypo- and hypertonic solutions employed throughout this work were distilled water and Ringer's solution made up with buffer and in concentration that is described as isotonic for amphibian tissue.

The effect of distilled water on the weight of *Planaria dorotocephala* and the analysis of the distilled water used are given in a former paper (Buchanan, 1930*a*). It was also shown that these animals in some way condition distilled water; accordingly in this work the proportions of animal tissues to distilled water were made approximately the same in all experiments. Since the changes in weight in hypo- and hypertonic solutions constitute an important phase of the problem at hand, the method employed in obtaining the data given in the former paper is restated.

Twenty-five animals of medium length were weighed and placed in 250 cc. of distilled water, then weighed again after certain intervals. The hydrogen ion concentration of the distilled water was reduced by the addition of a minute amount of NaOH. The experiments were conducted at room temperature, 20° to 23°. Control lots were weighed after similar handling in tap water and the difference in changes in weight between the distilled and tap water lots is regarded as due to the effect of distilled water on weight. The uniformity of the results was seriously interfered with by the fact that the distilled water causes the animals to exude slime. It is difficult to remove this slime during the weighing process. Furthermore, partial drying on filter paper and the incidental exposure to air causes a withdrawal of water from animals that have taken up water from a hypotonic medium. Thus the data show wide variations in weight changes in distilled water. It is clear, however, that after four hours in distilled water, pH 6.8 to pH 7.6, the weight increases. Consequently for respiration tests I employed animals that had been pre-treated with distilled water for from four to six hours.

The effect of Ringer's solution on the weight of *Planaria dorotocephala* is also given in a former paper (Buchanan, 1930*b*). Ringer's solution was employed because in the writer's experience the animals live longer in this hypertonic medium than in others that have been tried. At 20° medium sized *Planaria* will live for several days in Ringer's solution. Data on the effect of Ringer's on the weight of the animals were obtained by methods similar to those employed for distilled water. The results are fairly uniform, for the animals do not exude slime. It is shown that the weight decreases continuously during the first four hours of exposure, but after that time further loss of weight is slight. Hence, for tests of the effect of loss of water from the animals on their rate of oxygen consumption under different conditions of oxygen concentration, I employed animals that had been exposed for four hours previously to Ringer's solution.

The oxygen content of the various liquids was determined by the Winkler method. This method has recently been criticised by Shearer (1930). His criticisms are based in large part on his statement that when present in numbers a European planarian discharges substances which should, according to Shearer, absorb iodine and thus interfere with the titrations. Shearer assumes that this possibility has not occurred to us. The fact is rather elementary that in iodometric work the possible absorption of iodine by substances in the solution must be taken into account. Years ago Hyman (1919) showed conclusively that the substances which *Planaria dorotocephala* excretes do not absorb iodine. Further evidence is to be found here. *Planaria* exude slime in distilled water but none at all in Ringer's solution, yet the titrations show that the oxygen consumption of the animals is materially less under comparable conditions in distilled water than in Ringer's.

Shearer also criticises work done with *Planaria* by this method on the grounds that the motor activity of the animals is not controlled. Shearer himself attempts to draw conclusions concerning the motor activity of an American flatworm which he probably has never seen, from quite unconvincing evidence obtained from a European form. If the head of *Planaria dorotocephala* is removed the animal continues to crawl about for some minutes. Then it comes to rest in a contracted state and if undisturbed does not change position for many hours. This is also true of *Planaria maculata* but not true of *Phagocata gracilis*. A comparison of the rate of oxygen consumption of beheaded *Planaria dorotocephala* with that of intact animals brought to rest by other methods, namely, lowering the temperature slightly and excluding light and other stimuli, shows no appreciable difference (Hyman, 1919*a*, 1919*b*; Buchanan, 1926). It has thus far been impossible in this, as in much of the work on standard metabolism, to exclude local muscular twitching and the action of cilia, without recourse to profound anesthesia. If the work on *Planaria* is invalidated by these internal and localized motor activities, then the same standards must be applied to a very considerable proportion of the work on respiratory metabolism, including Shearer's. In the present work the factor of motor activity of the animals is not seriously concerned, for the respiratory differences are of such nature and so conspicuously different that they cannot be explained on the basis of differences in motor activity.

The possibility of the salts in Ringer's interfering with the titrations required safeguarding. Since only the relative quantities of oxygen in the medium before and after the animals had consumed some of it were of major importance, the action of salts may be discounted so long as it permits a sharp end point. To assure the accuracy of titration, a casserole containing the same quantity of Ringer's as the sample to be titrated was placed beside the casserole containing the sample. To this Ringer's was added sufficient iodine to match the color of the sample. It was then cleared with sodium thiosulphate. This served as a check end point, and the sample was then cleared to the same state of decoloration as the check. The end point in Ringer's was quite distinct and it was found unnecessary to provide this check as a constant procedure.

A much more difficult problem was the control of motor activity so that standard conditions obtained in the several environments. In part this was solved by the fact that *Planaria dorotocephala*, when changed from light to darkness, or when changed to a somewhat lower temperature, remain quiet for some hours. In this work both of these methods were employed. In both tap and distilled water the factor of motor activity does not enter, for in both the animals were quiet throughout the tests, except for the very brief time at the start, when the animals were distributing themselves about the interior of the flasks. In Ringer's solution the animals writhe for some time, then become paralyzed, accumulating at the bottom of the flask in abnormal positions. After two hours or less only occasional spastic movements occur unless light is admitted or the flask agitated. Unquestionably such twitching results in the consumption of oxygen. If one may judge from observations on the extent of such localized muscular activity, the amount of oxygen required for this movement must be very small as compared with the total oxygen consumption. Furthermore, there is no evidence in the observations that the extent of such movements increases with the increase in oxygen concentration of the Ringer's. After careful observation of the behavior of the animals as compared with the oxygen consumption under the several conditions, one must conclude that the differences in oxygen consumption cannot be ascribed to differences in motor activity.

In carrying out a titration the quantity of clearing agent used in reaching the end point is in practice slightly in excess or else slightly under the exact quantity. Also in the necessary manipulations no two lots of animals can be treated in exactly the same fashion. In order to compensate for such variations in procedure that result in a spread in the results, duplicate lots of animals were carried simultaneously in all experiments and the results recorded are the average between them. The oxygen consumption of such duplicates varied in amount, sometimes rather widely. But with one or two exceptions both members were in the same direction from the control determinations and fell within ranges that are consistent with the general data.

If the oxygen concentration of the medium is reduced materially below saturation at room temperature, some oxygen will be absorbed during a two-hour experiment, from the air or from the wall of the flask, or by leakage through the connections. Likewise, if the oxygen concentration is higher than saturation at room temperature, oxygen will be lost during the experiment. Obviously, therefore, determinations of the oxygen content of flasks in which animals had been kept for two hours had to be corrected for changes in oxygen concentration not due to the metabolism of the animals. A considerable series of tests was made in an attempt to determine the magnitude of change at various oxygen concentrations. The apparent oxygen consumption of the animals was then corrected in each case in accordance with the average oxygen gain or loss at approximately the same oxygen tension in tap H_2O in the absence of animals. Admittedly this does not yield an absolutely accurate result, for the control tests just described showed that the amount of oxygen gain or loss is subject to some variation. However, this appears to be the most accurate and convenient method at hand for establishing the necessary correction. A tabulation of the average oxygen gain or loss at various oxygen tensions which was used as a table of corrections is given as Table III.

A typical experiment to test the effect of differences in osmotic pressure of the medium on the response of the organism to different oxygen concentrations was carried out as follows: Twenty-four hours before the test, if distilled water was to be used, a large quantity of distilled water was treated with carbon dioxide-free air, the water being treated for at least twelve hours. The H ion concentration was thus decreased to pH 7.0 prior to use in the experiment. A selected lot of animals was divided into two groups and their rates of oxygen consumption in tap water were taken over a two-hour period. In all cases the oxygen concentration of the tap water was at or near saturation at 20°; the H ion concentration being always lower than pH 7.0 but never as low as pH 7.8. The amount of oxygen consumed per two hours under these conditions is regarded as the normal and in comparison with the effects of other conditions is taken as 100 per cent. The animals were then placed in 500 cc. of distilled water, or of Ringer's, as the nature of the experiment required, the oxygen tension of which was approximately that of the tap water in the control test. After four or more hours a fresh supply with higher or lower oxygen tension, but with the same H ion concentration, was siphoned into the flasks, after appropriate washings, which were then sealed and immersed in the constant temperature bath in darkness. Samples for titration were taken of the stock solutions and of the contents of the flasks at the end of two hours.

The oxygen concentration of the stock solutions was controlled either by bubbling pure oxygen through the stock, or by driving out most of the oxygen by heating and then cooling in a container so sealed as to prevent the ingress of air directly to the supply to be used. The time intervals were accurate to within one minute, the temperature within 0.5°, and the H ion concentration ranged in the various experiments from pH 7.0 to pH 7.8. In the case of Ringer's the pre-treatment with carbon dioxide-free air was not necessary, since the solution was buffered to pH 7.8. In all cases the test containers were Erlenmeyer flasks, capacity 500 cc., in which were placed from 35 to 60 animals, depending on size. Over a two-hour period the products of the animals increased the H ion concentration of distilled water approximately pH 0.2, somewhat less in tap water, and not at all in Ringer's. Therefore at no time was the H ion concentration greater than pH 6.8, nor less than pH 7.8. According to Hyman (1925), within this range the effect of H ion concentration on oxygen consumption is slight.

DATA

Table I shows that the water content of *Planaria dorotocephala* is approximately 78 per cent of the total weight, as determined by drying over sulphuric acid. The accuracy of this value is of course conditioned by the weight of water adherent on the surfaces of the animals at the first weighing and by the distribution of water between the drying agent and the organic material. The affinity of the latter for water being undetermined, it is not justified to assume that all the free water is removed by this method. With these reservations, it is, however, certain that the water content of these animals is relatively low. Comparison of these data with those given by Davenport (1896, pp. 58–59) for other invertebrates shows that the water content of *Planaria* is comparable with that of other soft forms, although somewhat lower. It is much lower than that of hyaline forms referred to by Krogh (1916, pp. 58–59). Thus the observed facts do not support Krogh's hypothesis, that independence of rate of oxygen utilization of environmental oxygen supply is conditioned on a low content of dry substance.

The effect of distilled water and of Ringer's solution on the weight of *Planaria* is given in two former papers (Buchanan, 1930*a*, 1930*b*).

TABLE I

Loss of weight of Planaria dorotocephala over sulphuric acid. All animals starved two weeks before weighing. In milligrams and percentage change.

Length	No. of Animals	Live Weight	Final Weight	Loss
mm.		*mg.*	*mg.*	*per cent*
15	5	46.2	9.8	78
"	8	61.9	14.1	77
"	11	74.6	20.0	73
"	14	109.1	23.7	78
"	18	131.2	28.1	78
"	25	180.5	38.6	78
"	20	142.4	34.0	75
"	20	134.4	31.7	76
"	20	143.7	28.2	80
"	20 +	160.5	34.4	78
16	20	196.7	36.7	81
"	20	194.7	39.2	79
"	20 −	159.7	36.3	77
"	20 +	223.3	44.7	79

Table II is abstracted from the data there given and consists of weight changes in experiments in which the conditions were comparable to those which were employed in testing the effect of these environments on respiration. As stated above (p. 312), it was found impossible to weigh animals from distilled water with accuracy, due to the adherent slime and the accidental drying that occurs when the animals are handled on filter paper. The average weight gain shown by the data is approximately 15 per cent; the organism being 78 per cent water, this represents an increase in total free water of 11 per cent. No great confidence may be placed on these data, however. At best they merely indicate that water is taken up by the animal while in distilled water.

TABLE II

Changes in weight of Planaria dorotocephala after four- and five-hour treatment with Ringer's solution and distilled water. Abstracted from Table III, 1930*a*, and Table III, 1930*b*. All animals starved at least one week. 14–18 mm. animals. Twenty-five animals in 250 cc. of liquid in each case. Room temperature, Ringer's solution, pH 7.8; distilled water, pH 7.0 to pH 7.6. In percentage change as compared with controls in tap water.

Ringer's Solution		Distilled Water	
4 hours	5 hours	4 hours	5 hours
− 17.7	− 17.7	+ 1.9	+ 24.6
− 17.7	− 19.0	+ 3.7	+ 18.6
− 20.9	− 18.5	+ 4.5	+ 16.5
− 16.9	− 14.2	+ 1.9	+ 11.9
− 17.0		+ 0.6	− 2.8
− 10.0		+ 1.5	− 1.1
− 11.0			
− 12.0			
Average − 16%		+ 15%	

In the case of Ringer's, the data show clearly that the water loss amounts to approximately 16 per cent of the total weight, or a reduction of approximately 20 per cent of the total water content.

Figure 1 shows in graphic form the effects of changes in the oxygen tension of the medium on the rate of oxygen consumption in distilled water, tap water, and Ringer's solution. The points on the respective graphs were determined by consolidating the data as follows: For each oxygen concentration duplicate lots of animals were used and the average between them is set down as the result at that particular oxygen concentration. Up to 8 cc. of oxygen per liter the determinations within a range of 1 cc. of oxygen per liter were averaged and plotted. Above 8 cc. per liter determinations of oxygen consumption within each concentration range of 2 cc. per liter were averaged and plotted. The graphs, then, represent 52 separate determinations of oxygen consumption in distilled water, 54 determinations in tap water, and 68 determinations in Ringer's solution.

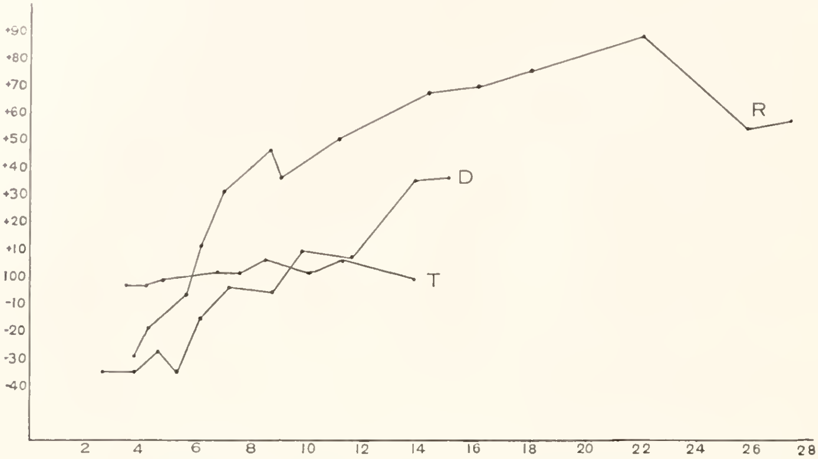

FIG. 1. The effect of increase in oxygen tension of the medium on the oxygen consumption of *Planaria dorotocephala*. Abscissæ represent cc. oxygen per liter. Ordinates represent changes in rate of oxygen consumption as compared with rate in tap water with oxygen concentration at or near saturation at 20°, which is taken as 100 per cent. *T*, animals in tap water; *D*, animals in distilled water; *R*, animals in Ringer's solution. Temperature, 20° ± 0.5°. H ion concentration, pH 6.8 to pH 7.8.

The graph of the controls in tap water, *C* in Fig. 1, shows that within the range of oxygen tensions employed the rate of oxygen consumption does not change materially. For a number of years, in connection with other problems, data have accumulated on the rate

TABLE III

Average change in oxygen concentration in sealed 500 cc. flasks of tap water immersed in a water bath at 20° for two hours. Corrections for the data plotted in Fig. 1 were taken from this table. Change in percentage.

Range of Oxygen Concentration	No. of Experiments	Average Change
cc./liter		*per cent*
20–30	12	− 0.9
15–19	8	− 0.6
10–14	16	− 0.5
7–9	24	− 0.2
6	4	0.0
5	8	+ 0.1
4	4	+ 0.2
3	12	+ 0.6

of oxygen consumption of *Planaria dorotocephala* in oxygen tensions varying around the saturation point of water at room temperature. The data are in terms of the oxygen consumed per unit of wet weight per unit of time. At hand are 282 determinations that were made under comparable conditions; temperatures were between 18° and 24°; 208 experiments were conducted at 20°. The calculations necessary to determine the relationship between rate of oxygen utilization and the oxygen tension of the water have been carried out.[2] The results are as follows:

Number of experiments, 282.
Highest temperature of an experiment, 24° (3 cases).
Lowest temperature of an experiment, 18° (9 cases).
Average temperature, 20°.
Oxygen concentration of the water:
    Extremes, 3.90 and 7.19 cc. per liter.
    Mean, 5.87 cc. per liter.
    Standard deviation, 0.64 ± 0.018.
Oxygen consumption, per gram per two hours:
    Extremes, 0.30 cc. and 0.64 cc.
    Mean, 0.37 cc.
    Standard deviation, 0.05 ± 0.0014.
Coefficient of correlation, oxygen consumption and oxygen tension,
    0.09 ± 0.04.

While a statistical treatment of data of this sort is not free from criticism, the fact that the coefficient of correlation is not significant, when considered together with the graphic results shown in Fig. 1, and the data of Hyman (1929), constitute convincing evidence that the rate of oxygen consumption of *Planaria* in natural water is independent of the oxygen tension over a wide range.

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[2] These calculations were made by Miss Rosalthea Sanders.

Figure 1 also shows that in distilled water after four to six hours the rate of oxygen consumption is sub-normal and remains fairly constant between oxygen concentrations of 2.65 and 5.35 cc. per liter.[3]

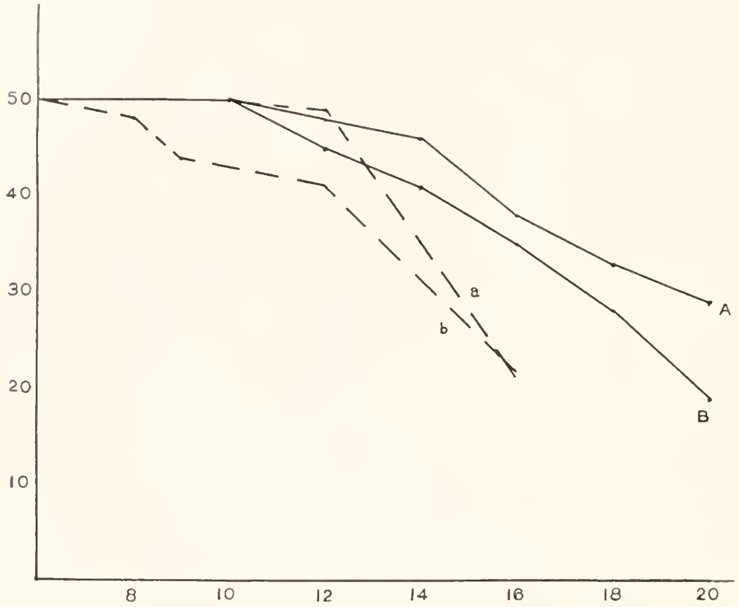

FIG. 2. Rate of disintegration of *Planaria dorotocephala* in distilled water with different oxygen content. Ten animals in 500 cc. distilled water in each case. Room temperature. Abscissæ represent hours; ordinates are derived from arbitrary values assigned to portion of animals alive at each observation, *e.g.*, 50 indicates that all 10 within a flask are alive; 40 indicates that some have partially disintegrated. *A* and *B* and *a* and *b* represent two different experiments. In *A* the oxygen concentration of the distilled water was 11.94 cc. per liter; in *B* the oxygen concentration was 6.16 cc. per liter. In *a* the oxygen concentration was 17.75 cc. per liter; in *b* the oxygen concentration was 6.51 cc. per liter.

It increases as the oxygen concentration is further increased, but is below that in tap water until the oxygen tension is increased beyond

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[3] There is an apparent discrepancy between these results for distilled water and those of Hess (1929) and those given in an earlier paper by the writer (Buchanan, 1929*a*). The depressive action of distilled water shown here arises from the fact that the animals were pre-exposed, after washing with distilled water, from four to six hours, then washed by siphoning distilled water through the flasks for several minutes, then sealed in distilled water for two hours, thus bringing the total exposure to distilled water to totals of six and eight hours, in large volumes. The earlier results (1929*a*) and those of Hess (1929) agree that long exposure to distilled water lowers the rate of oxygen utilization. The data here given are not in conflict with those results.

9 cc. per liter. In Ringer's solution the fact appears that the rate of oxygen consumption is sharply dependent on the oxygen tension of the solution until the tension reaches a high value, approximately 14 cc. per liter. The effect of increasing the oxygen concentration above 14 cc. per liter becomes increasingly less; the data indicate that in the highest concentrations employed the rate of oxygen consumption is somewhat less than in lower concentrations.

Thus it appears that although the animals in natural waters are independent over a wide range of oxygen concentration, this is not a fixed condition, but is susceptible of being materially affected by either the salt content of the water, or by the osmotic pressure, or by both.

Figure 2 shows that animals in distilled water with a high oxygen content disintegrate slightly less rapidly than do animals in distilled water with oxygen content around the saturation point at room temperature. The method of plotting these data has frequently been described in detail and will not be re-described here. Reference to Fig. 1 shows that the rate of oxygen consumption of animals in distilled water containing corresponding concentrations of oxygen is higher in the higher concentrations, the reverse of the order of disintegration. In other words, in high oxygen concentrations the rate of oxygen utilization is greater while the rate of disintegration is lower than in normal oxygen tensions.

Discussion

With regard to the original problem, what is the mechanism that renders the rate of oxygen consumption of this animal indifferent to oxygen concentration changes in the environment, these results render certain hypotheses improbable.

In the first place, it is clearly shown that this characteristic, in this particular animal at least, may be modified by the action of salts and by the osmotic pressure of the environment. As a matter of methodology, this fact is of considerable importance. For if one were to measure the oxygen consumption of an animal in Ringer's solution, for instance, with the oxygen tension slightly greater than saturation at 20°, the data would appear to show that Ringer's accelerates oxygen utilization. On the other hand, should the oxygen concentration of the Ringer's be somewhat under saturation at 20°, the results would indicate that Ringer's is a depressant. The effects of various salts on respiratory metabolism have received considerable attention, but there is little uniformity in the findings of various investigators. A partial review of the literature is given by Hess (1929).

Secondly, the results are important in showing that the absolute size of the animals, *i.e.*, the surface-volume relation, as suggested by Shoup (1929), may be rejected as a general factor in determining the relation between rate of oxygen utilization and oxygen tension. Also the rôle played by the degree of perfection of the respiratory and circulatory mechanisms does not appear to be of general importance; it is inconceivable that Ringer's or distilled water in any way converts these mechanisms in *Planaria*.

With these hypotheses of limited application, attention must be focused on the cellular oxidative mechanisms, not sufficiently emphasized in Krogh's (1916) discussion. The possibility must be considered that the relation between rate of oxygen utilization and the oxygen concentration of the environment is conditioned on the concentration of oxidative enzymes in relation to the oxygen concentration within the tissues. In the total absence of any information whatever as to the oxygen tension in the tissues, the permeability of the tissues to oxygen, and the effects of Ringer's solution and distilled water on tissue permeability to oxygen, explanations of the causal factors in these results must be in large part conjecture. However, sufficient facts are shown by the data to justify a discussion of certain possibilities.

In the normal animal in tap water, assuming that oxygen is present and oxidizable substances are in excess in the tissues, the velocity of oxidations is a product of a constant and the concentration of the active enzymes. That is,

$$\textit{Velocity} = kE.$$

So long as E remains constant, further increase in the oxygen tension should have no effect on the rate of oxygen utilization. This constitutes a possible explanation of the fact shown in Fig. 1, that increasing the oxygen concentration of the tap water has no effect on the rate of oxygen utilization within the concentration range employed.

But if the value of E is increased, thereby increasing the value of V, the oxygen concentration in the tissues becomes a limiting factor until it reaches a value equal to the oxygen-transferring power of the enzymes as conditioned by the rapidity of adsorption of the substrates. If the value of E is decreased, V is proportionately decreased and remains constant so long as the oxygen tension is adequate to maintain a constant adsorption rate.

The data on both Ringer's and distilled water offer some support for this interpretation, for it will be noted in Fig. 1 that when the oxygen concentration reaches a certain value the rate of oxygen consumption tends to become constant. In distilled water this value is low, between 2.65 cc. and 5.35 cc. per liter, as would be expected if the cell enzymes were diluted. It is unfortunate that exact data on the water increase in distilled water have not been determined with accuracy; a dilution of 11 per cent could hardly account for the lowered rate of oxygen utilization as compared with the rate in tap water. Still, there is no general law stating the relation between enzyme concentration and the rapidity of an enzyme reaction except that small amounts of an enzyme are relatively more effective than are greater concentrations. The data here are in accord with this, but their nature does not permit of further analysis in this direction.

The evidence is considerably more impressive in the data on the effects of Ringer's solution. The rate of oxygen consumption tends to become constant at approximately 14 cc. oxygen per liter, a result to be expected if the cell contents are materially concentrated. Reduction of water content in Ringer's is approximately 20 per cent. These suggestions regarding the effects of both distilled water and Ringer's solution on the oxygen utilization necessarily include the assumptions that the oxygen tension in the tissues is less than that of the surrounding medium and increases with increase in environmental oxygen concentration.

Under normal conditions, with environmental oxygen in excess of requirements, undoubtedly one of the conditions which determine the rapidity of oxygen utilization in the organism is the concentration of the oxidative enzymes. It is not unreasonable to suppose, and the results here support the view, that at least one of the conditions that determine the degree of dependence of oxidative rate on oxygen tension of the milieu is the relative concentration of the oxidative enzymes.

Another interpretation of lines *T* and *R* in Fig. 1 is possible. For this, two assumptions are necessary: (*a*) that in tap water with oxygen concentration between the minimum and maximum employed, the intra-cellular oxygen tension is greatly in excess of requirements, and (*b*) that Ringer's materially reduces permeability to oxygen, thus reducing intra-cellular oxygen tension. Under such conditions the rate of enzyme-controlled oxidations within the cell in tap water may be expected to remain constant with increase in environmental oxygen supply, while in Ringer's increasing the environmental oxygen concentration would be expected to effect corresponding increase in intra-cellular oxygen tension, with consequent increase in rate of oxygen utilization. The fact that in Ringer's the effects of increasing oxygen concentration form a logarithmic curve rising well above the rate in tap water must be referred to the effect of Ringer's in extracting water and by concentration increasing the rate of oxygen transfer by the enzymes.

However, the fact that the effects of increasing the oxygen tension in distilled water above 5.35 cc. per liter are in general parallel to the effects in Ringer's, in that the rate of oxygen consumption is no longer independent of the supply, seems to indicate that some condition common to both distilled water and Ringer's disturbs the normal oxidative mechanism. Apparently, the ability of the oxidative enzymes to transfer oxygen is not adversely affected, for in both the rate of oxygen utilization increases above the controls with increase in oxygen concentration. Since in time both distilled water and Ringer's are lethal, it is conceivable that this common effect is an indication of the onset of structural breakdown. The most probable early injurious effect that would bring about this common effect is interference with the normal cell permeability to oxygen. While permeability effects are usually referred to the cell boundary and there is substantial evidence that the cell boundary does regulate the admission of oxygen (Warburg, 1911), permeability of the general cytoplasm must also be considered.

An interesting fact appears in the data on the effect of increasing oxygen tension in Ringer's solution. When the oxygen concentration of the Ringer's is approximately at saturation for that concentration of salts at 20°, the rate of oxygen consumption is approximately that of normal animals in natural water. Oxygen being less soluble in Ringer's than in water at the same temperature, this saturation point therefore represents an environmental oxygen supply somewhat less than at saturation in tap water. The significance of this fact is not apparent; it may be merely incidental with this particular concentration of salts.

Figure 2 shows that oxygen in the concentrations employed is not toxic for *Planaria*. The animals disintegrate slightly less rapidly in abnormally high concentrations of oxygen in distilled water than in distilled water with oxygen tension around the saturation point at room temperature. Also, it may be pointed out, with reference to Fig. 1, that in distilled water at oxygen concentrations corresponding to those used in Fig. 2 the rate of oxygen consumption is higher in the higher concentrations, while Fig. 2 shows that the rate of disintegration is slightly lower in the higher oxygen concentrations. In other words, the higher the concentration of oxygen the less rapid the rate of disintegration and the higher the oxygen consumption. These facts substantiate the conclusion expressed in a former paper (Buchanan, 1930*a*), that disintegration in distilled water is not a direct result of the action of the hypotonic medium on the oxidative metabolism of the animal; that relative rate of disintegration in distilled water is not an index of relative rate of oxidative metabolism.

SUMMARY

In tap water the rate of oxygen consumption of *Planaria dorotocephala* is independent of the oxygen concentration of the water between 3.53 cc. and 13.84 cc. per liter. In so far as the methods coincide, this confirms the results of Hyman (1929).

In Ringer's solution after four hours' exposure *Planaria* lose approximately 16 per cent of their total weight, or approximately 20 per cent of their total water content. In Ringer's the rate of oxygen consumption is sharply dependent on the oxygen concentration of the solution between 3.79 cc. and approximately 14 cc. per liter. Above this concentration the rate of oxygen consumption tends to become constant.

In distilled water the animals imbibe water; irregular data indicate that during the first four or five hours of exposure the weight increases on the average 15 per cent, which represents an increase in water content of approximately 11 per cent. The rate of oxygen consumption in distilled water is lower than normal and constant in oxygen concentrations between 2.65 cc. and 5.35 cc. per liter. Above this concentration the rate of oxygen consumption increases as the oxygen tension increases, rising above the normal at approximately 9 cc. per liter, and continues to increase as the oxygen concentration increases, up to 15 cc. per liter, the maximum employed.

Planaria dorotocephala contain approximately 78 per cent free water, as shown by weight loss on desiccation over sulphuric acid.

The facts indicate that two conditions may be involved in the regulation of the rate of oxygen utilization in relation to oxygen tension in natural water: Concentration of water within the organism, thus controlling the concentration of the oxidative enzymes; boundary regulation of the admission of oxygen. In this animal, size (surface-volume) and the degree of development of its circulatory and respiratory mechanisms do not appear to be important.

In abnormally high oxygen tensions in distilled water the rate of oxygen consumption is higher and the rate of disintegration is lower than in distilled water with oxygen tension at saturation at 20°.

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OBSERVATIONS ON *EUGLENA LEUCOPS*, SP. NOV., A PARASITE OF *STENOSTOMUM*, WITH SPECIAL REFERENCE TO NUCLEAR DIVISION[1]

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Introduction

A *Stenostomum* heavily infected with a colorless euglenoid parasite was found in a collection made near the University of Virginia. The morphology of this curious flagellate has led me to place it in the genus *Euglena* Ehrbg.

My purpose is to describe this new form, its nuclear division and its relation to its host; discuss its systematic position; and record some further observations upon its life history.

Historical

Euglena-like parasites of rhabdocoels have been reported on previous occasions. Haswell (1892) reported from an undetermined species in Australia an intracellular, colorless flagellate that had neither stigma nor flagellum. In 1907 the same author reported from the same continent another colorless form, from a mesostomid. It possessed a stigma and when liberated from the host, it no longer progressed by metabolic or euglenoid movement but became somewhat bottle-shaped and swam spirally by a flagellum. This parasite was found not only inside the cells but also in the space between the gut and body wall. He did not attempt to identify either of these forms.

In France, Beauchamp (1911) described *Astasia captiva*, a parasite within the "pseudocoele" of *Catenula lemna*. His description is fairly complete. This form retained its flagellum in the host and bore a colorless "rudimentary" stigma. It measured 30 to 40 microns in length and even when liberated from the host moved only by a rapid metabolic (euglenoid) movement. He speaks of, and his figures show, the oblique surface striations and spoon-like depression near the posterior end of the body. He mentions the "conduit buccal" and nucleus as being typical of the "Eugleniens."

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[1] The writer is greatly indebted to Dr. B. D. Reynolds, under whose direction this investigation was carried out at the Miller School of Biology and the Mountain Lake Biological Station of the University of Virginia.

MATERIALS AND METHODS

The infected *Stenostomum* (a new species soon to be described) was found in one of my aquaria in October 1929.[2] Although there were several other *Stenostoma* of the same, as well as of other species, in the aquarium, none were found to be infected. No free-living flagellates were found in the water that resembled the parasite. Other old aquaria in the laboratory as well as fresh collections were then examined. Of the hundreds of rhabdocoels examined, only two species were found infected and these were both species of *Stenostomum*. The other *Stenostomum* found infected is *S. predatorium* (Kepner and Carter, 1930). The infection is very rare in nature.

Fortunately, one of these *Stenostoma* was cultivated very easily in rather large glass dishes in which there were wheat cultures of small flagellates and ciliates. A half dozen of these flatworms added to a thriving protozoan culture will produce a hundred or more in two or three weeks. The worms were examined frequently to see if any of the free-living Protozoa had become established as parasites. Negative results are reported. Upon the addition of two or three infected *Stenostoma*, practically all the flatworms were infected in a week. In this way it was possible to have on hand as many specimens of *Euglena leucops* as were desired.

Various killing and fixing agents were employed. The best fixation of the chromatin was obtained with Carnoy's aceto-alcohol mixture. Absolute alcohol saturated with corrosive sublimate gave good results, as did Bouin's picro-formol solution, Allen's modification of the former, Schaudinn's and Zenker's fluids. Nuclear division was best studied in sectioned hosts, although smear preparations were also used. Heidenhain's iron alum haematoxylin was found to be the best stain for the nucleus, flagellum and blepharoplasts. The alcohol-xylol-paraffin method was employed.

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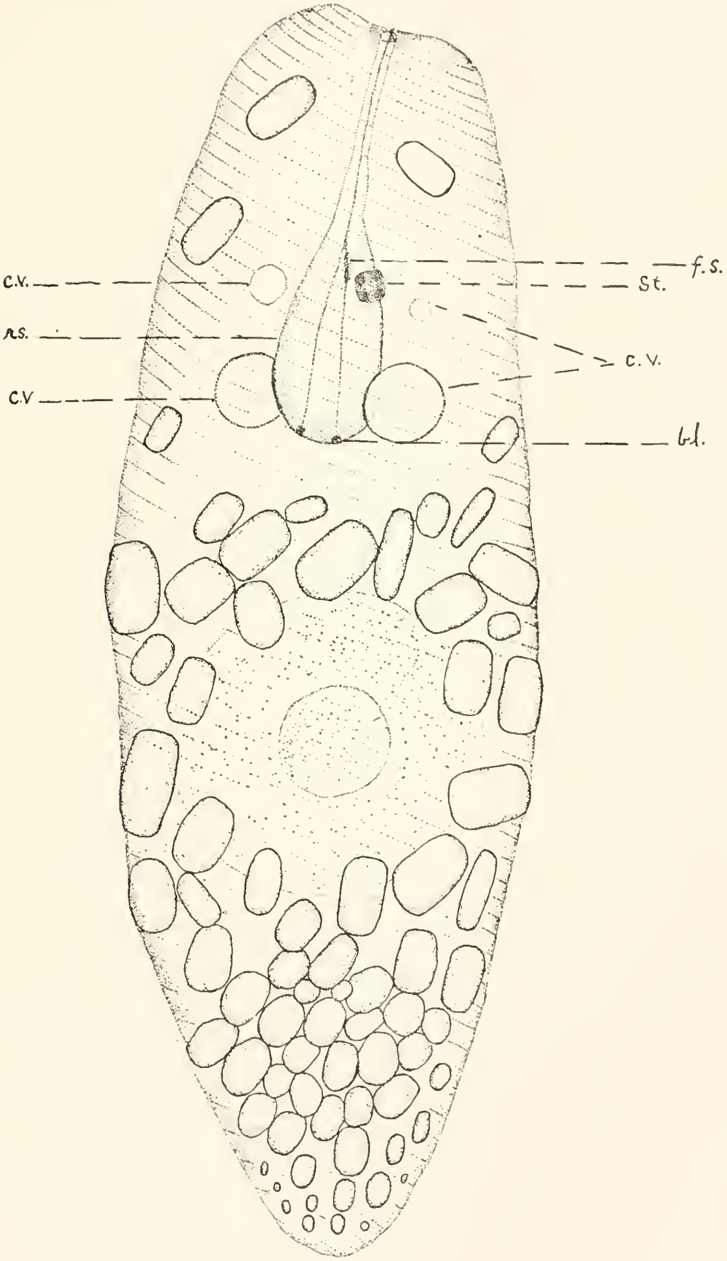
[2] I am greatly indebted to Dr. W. A. Kepner for help in the identification of the rhabdocoels employed in this investigation.

PLATE I (× 8,000)

FIG. 1. Drawing of a non-flagellated living specimen of *Euglena leucops* sp. nov. as it appears immediately after liberation from the host.

The two rami of the flagellum can be seen to end at the blepharoplasts (*bl.*) at the base of the reservoir (*rs.*). The flagellar swelling (*f.s.*), a little anterior to the stigma (*st.*), is barely visible. The oblique surface striae are shown as well as both face and side views of the paramylum grains. Four contractile vacuoles (*c.v.*) are figured, two extended and two contracted. Details of the living nucleus other than the endosome can not be clearly made out. The stigma illustrated is composed of four distinct particles.

Plate I


f.s.
St.
c.v.
r.s.
c.v.
c.v
b.l.

General Morphology

The organisms vary as to size. In a contracted condition the range in length is from 22 to 29.6 microns. Elongated, the range is from 33 to 44.5 microns. In width, the range is from 9 to 13 microns for resting individuals. In a resting condition the parasites are approximately two and one-half times as long as wide and rather blunt on both ends. The anterior end bears a slight depression in its contour where the gullet has its outlet. Most of a population are of average size. The forms just before cell division present the maximum size, but the daughter cells produced are often larger than others seen. The wave formed during metabolic movement may be as great in diameter as 18.5 microns. During metabolic movement the anterior end decreases only slightly in diameter while the posterior end becomes quite pointed.

Very fine surface striae run obliquely from left to right (Plate I, Fig. 1).

There are no chromatophores in the cell.

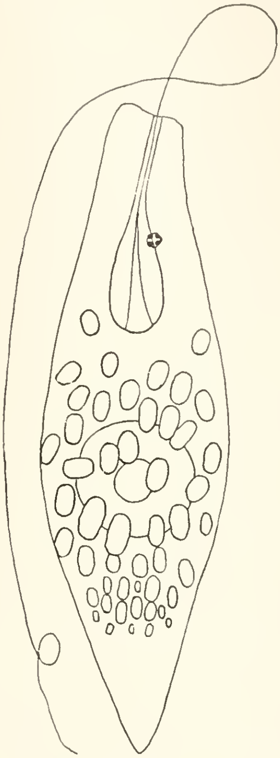
A gullet and reservoir can be seen in the living animal. The two together present a flask-shaped outline with a narrow passage, the gullet leading anteriorly. Associated with the reservoir are a series of contractile vacuoles that open into it. As many as four vacuoles have been counted in living specimens (Plate I, Fig. 1).

Flagellum

When the parasite is in the host the flagellum does not extend beyond the opening of the gullet (cytostome). Even in living individuals the flagellum can be seen to bifurcate as soon as it has passed the narrow passage of the gullet, the two rami proceeding to the blepharoplasts at the bottom of the reservoir. There is a "flagellar" swelling at the point of bifurcation, which is approximately at the level of the stigma (Plate I, Fig. 1).

When the parasite is liberated from the host and put into tap or spring water, a flagellum can be seen beginning to grow out of the "stump" in less than ten minutes. In twenty-five or thirty minutes in this medium it has reached its maximum length, a little longer than the body. Even before it has attained a length one-half as great as the body, the parasite ceases its metabolic movement and progresses feebly by the flagellum. In this condition the organism is dumb-bell-shaped, moving forward irregularly in very small spirals. The cell is still blunt on the posterior end at this stage. By the time the flagellum has reached its full length, the shape of the cell has changed greatly. It is now bottle-shaped, moving vigorously and swiftly by means of the newly acquired organelle of propulsion (Text figure 1).

The animal displays a marked faculty to replace a lost flagellum, since, as pointed out below, this structure when lost will be regenerated if the conditions of osmotic pressure are made favorable.



Text Fig. 1. Outline drawing of a flagellated specimen of *Euglena leucops* sp. nov.

Paramylum Bodies

These bodies are distributed irregularly over the cell, being most abundant in the posterior third. Only one to three small ones may be seen anterior to the stigma (Plate I, Fig. 1). These granules sometimes measure 4 microns at the greatest diameter. They are found to be disc-shaped and to decrease regularly when the parasite is subjected to inanition.

Stigma

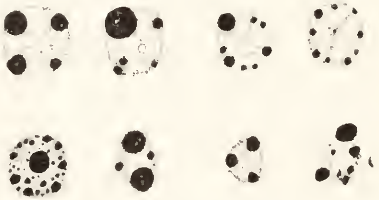
The stigma, dark reddish-brown in color, stands out distinctly in the anterior region, just lateral to the gullet. It is not large, measuring less than two microns at the greatest diameter.

Much variation in size and form of the stigma has been observed (Text figure 2). The stigma is seen to be composed mainly of four to seven distinct bodies which appear to be connected by fine strands and the whole embedded in a colorless matrix. This morphologically degenerate stigma may represent a stage in the complete loss of this organelle.

A large number of observations were made upon the stigma of dividing individuals. While the nucleus is in the early prophase, the stigma enlarges slightly but usually the number of colored bodies of which it is mainly composed does not increase. These granules push apart somewhat and, keeping their identity, usually about one-half of them migrate, probably attached to each other, to the opposite side of the cell, never wandering far from the reservoir. They finally come to lie in a position lateral to the new gullet which has formed in the meantime.

This account of the origin of the daughter stigmata agrees somewhat with the observations of R. P. Hall and Jahn (1929*b*) on *Euglena gracilis*, except that they found that the stigma breaks up into a large number of smaller granules which become more widely separated.

The stigma does not always divide equally. A number of times the daughter cell has been seen to receive only two-fifths or even one-fourth of the dark granules. This unequal division aided by natural selection perhaps accounts for the decreased size and degeneration of the stigma.



TEXT FIG. 2. Variations in the stigma of *Euglena leucops* sp. nov. Drawings from several specimens. × about 16,000.

Nucleus

The nucleus can be seen in the living organism as a relatively large spherical body (8 to 9 microns in diameter) lying usually near the center of the body. Upon fixing and staining, its chief characteristic is the presence of usually one comparatively large, deeply-staining, central body, the endosome (karyosome of some authors). Immediately surrounding the endosome is a hyaline area, apparently lacking in chromatin. The twenty-two to twenty-five chromatin bodies which have been called "chromosomes" by Keuten (1895) and which give rise to the same number of chromosomes, remain distinct even in the nucleus of the interphase or the resting nucleus. Their distal ends lie close to and probably join the well-defined nuclear membrane. Fine strands are distinctly seen connecting the proximal ends of these bodies with the endosome (Plate II, Fig. 1).

These observations on the chromatin in the resting nucleus are not in agreement with those of most other workers on the euglenoids. Numerous small granules arranged at the nodes of a linin network have been described by Hartmann and Chagas (1910) for *Peranema*, by Tschenzoff (1916) for *Euglena viridis*, by Bělař (1916) for *Astasia*, by R. P. Hall (1923) for *Menoidum*, by R. P. Hall and Powell (1928) for *Peranema*, by Baker (1926) for *Euglena agilis* and by Ratcliffe (1927) for *Euglena spirogyra*. It is not difficult to see how the "numerous small granules" idea became prevalent, since this impression is frequently given by the nucleus after a casual observation, particularly following fixation in Schaudinn's fluid, the usual method employed. What I take to be the correct condition of the chromatin is better followed after the use of Carnoy's fixative. I have been able to observe the distinct "chromosomes" in the resting nucleus of *Euglena leucops* following fixation with all the agents mentioned in the section on materials and methods.

My observations are supported by Keuten (1895), who has figured chromosomes in the resting nucleus and in the late telophase for *Euglena viridis*. R. P. Hall (1925) has described the nucleus of the dinoflagellate *Oxyrrhis marina* thus, "Around the endosome the chromatin appears in the form of chromomeres, arranged in string-of-beads fashion in rows, or chromosomes; such an organization seems to be evident even in the resting nucleus, as characteristic of the dinoflagellates 'where there appears in the nuclei . . . to be a persistent organization of beaded chromosomes with subparallel or even spiral arrangements within the nucleus.'"[3]

In preparations fixed with Schaudinn's fluid and stained with iron haematoxylin, the endosome resists destaining very much longer than the chromosomes. After Carnoy's fixative and iron haematoxylin, the endosome decolorizes more readily than the chromosomes. The ability to destain the endosome almost completely has made possible a careful study of the resting nucleus and division stages.

A nuclear membrane is distinguishable in the resting condition and can be followed through mitosis.

Frequently, the endosome is seen to be in two, three or more fragments. R. P. Hall (Hall and Powell, 1928) noticed a multiplication of the endosome in *Peranema trichophorum*. Wenrich (1924), in his description of *Euglenamorpha Hegneri*, says: "In the pellucid variety there is a marked tendency for the nucleus to hypertrophy. . . . This hypertrophy is accompanied by a multiplication of the caryosome, as many as four have been found in one nucleus. Hypertrophy apparently leads to amitotic division of the nucleus which is probably followed by division of the body. Such amitotic stages have not been found in the green variety." No explanation is offered here for fragmentation of the endosome occurring in *Euglena leucops*. However, no evidence whatever for amitosis was found.

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[3] Hall is quoting from Kofoid (1923).

BEHAVIOR

Euglena leucops is stimulated to extremely vigorous movement by light, heat, and a change either way in osmotic pressure.

Thigmotropism is exhibited in non-flagellated individuals. Flagellated organisms were found to be negatively phototropic.

PLATE II (× 10,000)

All the figures were drawn from specimens fixed in the host with Carnoy's aceto-alcohol mixture and stained with Heidenhain's iron alum haematoxylin.

FIG. 1. A typical nucleus of the interphase. Twenty-two chromatin bodies or "chromosomes" are figured. Note the attachment of these to the endosome.

FIG. 2. A very early prophase. The "chromosomes" and endosome are beginning to enlarge.

FIG. 3. The nucleus is becoming noticeably larger and the "chromosomes" and endosome are beginning to elongate.

FIG. 4. Note the blunt bifurcation of one end of the endosome.

FIG. 5. The nucleus, chromosomes and endosome are all distinctly elongated. Most of the chromosomes have apparently lost their attachment to the endosome.

FIG. 6. This is the first stage in the metaphase. The chromosomes are quite narrow and extend the length of the elongate nucleus. The blunt bifurcation of one end of the endosome is distinctly pronounced by now and both ends have become enlarged.

FIG. 7. Metaphase and the beginning of the anaphase. The chromosomes, having spun out practically the length of the elongated nucleus during the early metaphase, now divide transversely as the extremities of the nuclear membrane and endosome further pull apart. Note the constriction in the membrane and endosome. Fine strands are seen in the mitotic figure at this stage, most of which appear to be a connection between the two broken ends of the chromosomes.

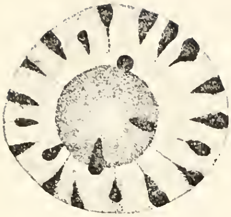
FIG. 8. The chromosomes are beginning to round off somewhat and are apparently attracted toward the two ends of the membrane. The nucleus and endosome are now decidedly elongated and constricted in the center.

FIG. 9. The chromosomes are beginning to assume their interphase shape and their attachment to the ends of the dividing endosome again becomes evident. Notice the upward turn of the nuclear membrane and endosome.

FIG. 10. The end of the telophase. Most of the chromosomes have regained their attachment to the endosome. The daughter nucleus and endosome now round off as the interphase approaches.

I am indebted to Miss M. E. Hill for help in the preparation of the drawings.

PLATE II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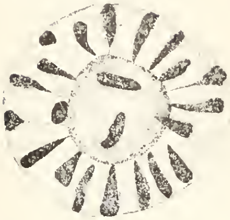
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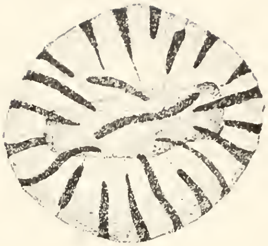
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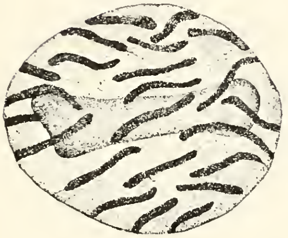
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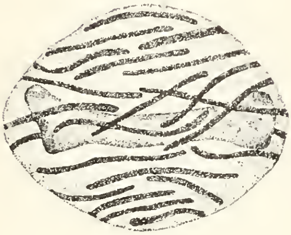
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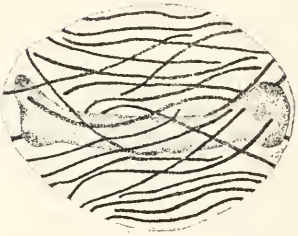
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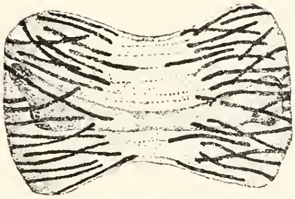
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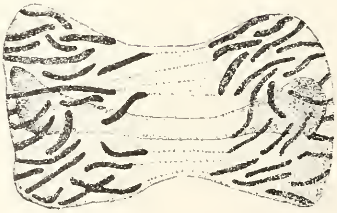
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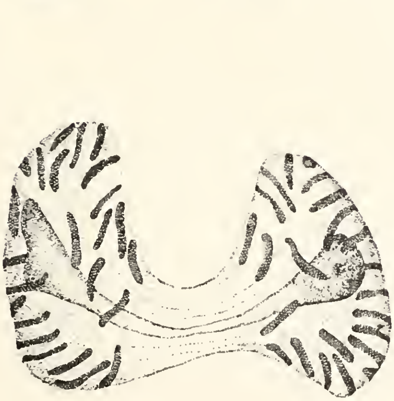
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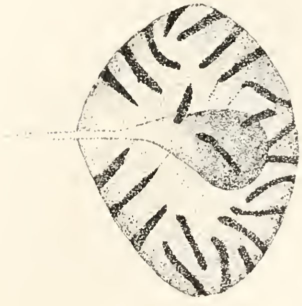
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The fact that *Euglena leucops* elaborates a flagellum soon after being liberated from the host and is negatively phototropic,—the host reacts similarly—makes it particularly well adapted for swimming away and finding a new host.

DIVISION OF THE BODY

When an infected host is macerated in spring water, at any hour of the day, numerous dividing parasites are encountered after a few minutes. Frequently as many as 10 per cent of the flagellates, after ten to fifteen minutes exposure to this solution, will start dividing and complete the process within seventeen to twenty minutes. Now if an infected host is macerated in a solution approximately isotonic with the host's plasma, no more dividing forms are seen in this medium than normally appear in the host and twenty-five to thirty minutes are required for the parasite to complete the division. Since the spring water is of a lower molecular and ionic concentration than the host's plasma, it would appear that the change in osmotic pressure both initiates and accelerates cell division.

Division in *Euglena leucops* seems to be periodic, as is the case in *Hydramoeba hydroxena*. Reynolds and Threlkeld (1929) observed that division in this amoeba occurred most often about 3 A.M. Observations upon more than three hundred dividing forms of *Euglena leucops* have shown that division, while sometimes occurring at other times, most often takes place between 10 P.M. and 2 A.M., with the peak about midnight.

A short time before the longitudinal split occurs, there is a duplication in the number of gullets, reservoirs, flagellar "stumps," stigmata, and nuclei. The split begins at the anterior extremity and continues posteriorly along the median plane of the body until the two daughters are completely separated. There is a continual twisting and writhing of the binucleate body during the process of division. Finally the posterior ends of the daughter cells are connected only by a small strand, but this is apparently the toughest part of the cell, for a veritable tug of war ensues, before the daughter cells are separated.

The parasite has been under observation in the host for more than a year and no evidence of encystation has been observed.

NUCLEAR DIVISION

Prophase

The beginning of nuclear division may be recognized in four ways: (1) increase in size of the nucleus, (2) migration of the nucleus anteriorly, (3) changes in the endosome and (4) changes in the chromatin bodies or "chromosomes."

A considerable increase in the size of the nucleus is observed at the onset of division.

By the end of the prophase the dividing nucleus has reached a position in the anterior part of the organism, just posterior to the reservoir.

The endosome increases in size and elongates. One end can be distinguished from the other by a blunt bifurcation (Plate II, Figs. 4, 5, 6, 7, 8 and 9). Baker (1926), working with *Euglena agilis*, describes the bud arising from one of the lateral points, as forming the blepharoplast of one ramus of the new flagellum and the connection of the bud with the endosome as the rhizoplast. I find insufficient evidence from *Euglena leucops* to support this.

By the end of the prophase the endosome is distinctly dumb-bell-shaped (Plate II, Fig. 5). It would appear that at the end of the prophase there is a brief pause in mitosis since this stage was encountered most often.

The distinct, rather large chromatin bodies or "chromosomes" of the resting nucleus, keeping their identity, increase slightly in size and stain more deeply early in the prophase (Plate II, Fig. 2). As the nucleus and endosome elongate, these bodies, with one end apparently remaining attached to the nuclear membrane, are spun out into extremely long chromosomes which become arranged around the endosome (Plate II, Fig. 5). The connection between the chromatin bodies or "chromosomes" and nuclear membrane persists certainly to middle prophase and perhaps until division is completed.

Metaphase

The endosome continues to elongate, its ends increasing in size at the expense of the middle portion (Plate II, Fig. 6). One end still can be distinguished from the other. The nuclear membrane follows largely the contour of the endosome. The chromosomes, having drawn out almost the length of the elongated nucleus, now break in the middle.

There is apparently little doubt that the chromatin bodies of the resting nucleus give rise directly to the same number of elongated chromosomes that divide transversely. Twenty-two to twenty-five bodies in the resting nucleus can be followed through the metaphase when the long chromosomes produce a double number of daughter chromosomes of approximately half the length of those in the late prophase (Plate II, Fig. 7).

I have been unable to find the V-shaped chromosomes of the late prophase that have been described for euglenoids by R. P. Hall (1923, 1928), Baker (1926), and Ratcliffe (1927) which they take as evidence for a longitudinal split.

After a review of a large part of the literature on mitosis in the Protozoa, I have been able to find only one other case where a transverse division in the chromosomes has been definitely demonstrated, although others have mentioned it as a possibility [Bělař (1916) in *Astasia* and Borgert (1909, 1910) in *Aulacantha* and *Ceratium*]. Calkins (1929) describes a transverse division in the chromosomes of the ciliate *Uroleptus Halseyi* on the third meiotic spindle.

Anaphase

During this stage the ends of the endosome become quite far apart but remain connected by their narrow, middle portion. The bend is quite pronounced, the two ends of the endosome making a sharp upward turn.

The forty-six to fifty daughter chromosomes now shorten but remain radially arranged around the extreme ends of the daughter endosomes. The membrane has become distinctly constricted in the middle largely following the contour of the endosome (Plate II, Fig. 8).

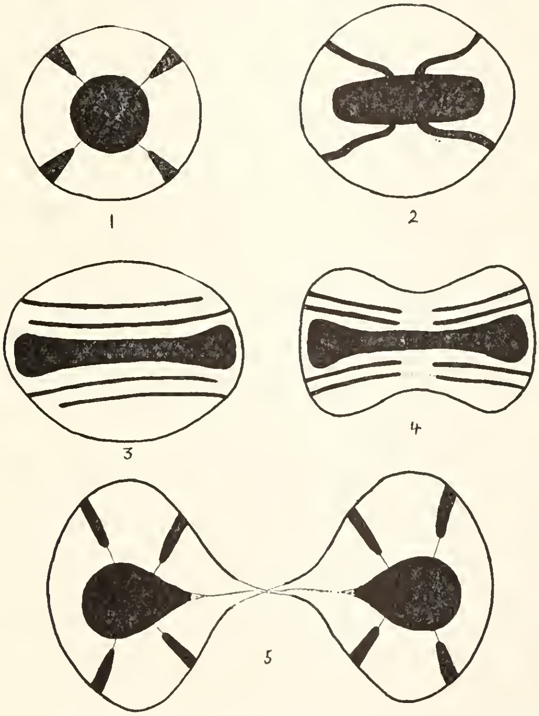
Telophase

This stage begins when the ends of the daughter endosomes and daughter nuclei have pulled apart. The original endosome breaks in the center of its now narrow, lightly-stained, central portion.

The daughter endosomes and nuclei now round off and the chromosomes arrange themselves at the periphery of the nucleus. The connection between chromosomes and endosome is established early in the telophase (Plate II, Figs. 9 and 10).

The accompanying diagram (Text figure 3) illustrates the history of four chromosomes, in the resting nucleus and through mitosis. Kater (1926, 1927, 1928) brings forth evidence that even in the higher plants and animals, reconstruction of the daughter nuclei is by vesicle formation of contiguous chromosomes, that may be traced well into the succeeding prophase. He considers this as "good evidence for genetic chromosomal continuity." The evidence for this continuity is still stronger in *Euglena leucops*.

Calkins (1929) has the following to say concerning the chromosomes of the ciliate *Uroleptus Halseyi*: "In *Uroleptus Halseyi* the chromosomes are divided transversely at each vegetative division. On the theory of the gene these facts are possible only on the assumption of a single type of gene for each chromosome and on this assumption there would be not more than twelve types of genes in *Uroleptus Halseyi.* Here there are twelve chromosomes in the third meiotic spindle and twenty-four in the amphinucleus. There are forty-eight in the first meiotic spindle and twenty-four in the second. We conclude that each of the twenty-four chromosomes found in the amphinucleus divides once to form the forty-eight of the first meiotic spindle; that two of these are separated from two by this division, and that one is separated from one at the second meiotic division, thus leaving twelve in the third meiotic spindle in which each chromosome represents a single type of gene. It is immaterial, therefore, whether division of each chromosome is transverse or longitudinal,



Text Fig. 3. Diagrammatic representation of the resting nucleus of *Euglena leucops* sp. nov. and the history of four of its chromosomes during division.

Fig. 1—the resting nucleus.

Fig. 2—the middle prophase.

Fig. 3—the beginning of the metaphase.

Fig. 4—the end of the metaphase and the beginning of the anaphase.

Fig. 5—the late telophase wherein begins the reconstruction of the nucleus of the interphase.

See the text and Plate II for a more detailed description.

for it would be equational in either case." Carrying this theory a step further, since the chromosomes are distinct even in the resting nucleus in the case of *Euglena leucops*, a single type of gene can be considered as recognizable at any stage in the life of the organism.

EXPERIMENTS ON RETARDATION IN GROWTH OF THE FLAGELLUM

As already stated, the flagellum of organisms liberated into tap or spring water will make its appearance outside the gullet in five to ten minutes. In 12 per cent Locke's solution, the organisms have been carried fourteen days without ever elaborating a flagellum. In a pure glucose solution with a molecular concentration approximately equal to the Locke's solution they have been carried more than a day or until death without showing a flagellum. The same thing is true for creatine, a mixture of different amino acids and other soluble protein derivatives. By varying the concentration of the molecular or ionic substances it is possible to vary the time required for the flagellum to be elaborated and for the appearance of the changes in body form that accompany it. The hydrogen ion concentration apparently has nothing to do with the retardation in the development of the flagellum. Viscous substances such as thin starch paste, as well as aqueous agar mixtures, which of course do not alter the molecular concentration of the media appreciably, have little or no effect on retarding the growth of the flagellum.

By increasing the osmotic pressure of the medium in which *Euglena leucops* elaborates and retains the flagellum, the organisms have been made to settle down and again move only by metabolic movement, the flagellum apparently being broken off. But if the osmotic pressure be once more decreased, the flagellum will reappear. This process has been repeated four times with the same individuals and the organelle grew out as readily the fourth time as it did the first.

It would appear therefore, that molecular or ionic concentration is the main factor in retardation of flagellar growth and that this organelle is capable of continued or repeated growth.

The osmotic pressure of the plasma of the host is apparently great enough to prevent the elaboration of a flagellum.

RESULTS OF ATTEMPTS TO CULTIVATE THE PARASITE

All attempts to cultivate the parasite *in vitro* failed. They were carried fourteen days in Locke's solution diluted to 12 per cent at pH 7.6. At the end of this period practically all the paramylum was consumed.

They may be kept alive three or four days in spring water.

METHODS OF INFECTION

The parasite may enter the host in three ways:

(1) By the ingestion of liberated parasites. The *Stenostoma* that the writer has been able to infect, feed extensively upon Protozoa. The parasites, either swimming actively or moving on the bottom of the container by euglenoid movement, are ingested by the host and enter the space between the gut and body wall, presumably, directly through the wall of the enteron.

(2) The *Stenostoma* susceptible to infection are predatory and cannabalistic. On a number of occasions I have observed the transmission of the infection by an infected flatworm serving as food.

(3) Vegetative zoöids are infected from the parent.

THE EFFECT OF THE INFECTION UPON THE HOST

Upon one occasion the writer observed an uninfected *Stenostomum* ingest a portion of an infected one of the same species. About eight parasites entered the enteron of the uninfected rhabdocoel. In less than an hour approximately all of the parasites were seen in the mesenchyme of the new host. The next day they had about doubled in number. The host divided on the second day and the (approximately thirty) parasites were distributed about equally between the two. At the end of a week the infection had become well established.

Stenostoma lightly infected show no ill effects whatever and appear normal in all their reactions. No signs of "nervousness" are exhibited even though the parasites can be seen passing over, under and in contact with the cephalic ganglia. Kepner and Carroll (1923) found this to be true in the case of *Stenostomum leucops* infected with the ciliate, *Holophyra virginia*.

Since the parasites evidently absorb their food from the plasma in the mesenchyme, one would expect that rather heavily infected animals would grow and reproduce slowly; but specimens infected with as many as fifty or sixty parasites have been seen to divide every second day under favorable conditions. This is as fast as normal individuals reproduce.

Frequently the flatworms become infected with two or three hundred parasites which causes them to appear bloated, due to the ectoderm being lifted to make room for the increased contents of the mesenchyme. Animals in this condition become sluggish, the ectoderm breaks in one or more places, the parasites are liberated and death generally follows in a day or so.

Means for freeing the flatworms of the parasites have not been found.

INFECTION EXPERIMENTS

Even though species of *Stenostoma* other than the two mentioned above ingest the parasite, only one, *S. grande*, has been infected experimentally. All attempts to infect *Catenula lemna*, the host of *Astasia captiva*, failed, even though it ingested *Euglena leucops* in large numbers.

SYSTEMATIC POSITION OF PARASITE

The apparent specificity of this parasite excludes the probability of its being found in hosts other than rhabdocoels. The only euglenoid described as a parasite of this group is *Astasia captiva*, from *Catenula lemna*, and this rhabdocoel apparently is not capable of becoming infected with *Euglena leucops*. *A. captiva* differs further from *E. leucops* in the following respects:

(1) *A. captiva*, according to Beauchamp (1911) is without a stigma, save possibly a *colorless* rudiment.
(2) It has a flagellum while in the host.
(3) It does not change in shape or otherwise when freed into spring water.
(4) *A. captiva* dies in a few hours after liberation.
(5) The paramylum grains of Beauchamp's parasite are elliptical in outline.

The *Euglena*-like form that Haswell (1907) mentioned but did not describe, resembles very closely *Euglena leucops*. Nevertheless it was intracellular and a parasite of a mesostomid, the most highly specialized of the Rhabdocoelida, and hence the farthermost removed from *Stenostomum*.

Wenrich (1924) has shown that the colorless variety, *pellucida* of *Euglenamorpha hegneri* may arise from the green variety, simply by loss of color. More conclusively Zumstein (1900) and Ternitz (1912) have shown that *Euglena gracilis* will lose its chlorophyll and become colorless when supplied with rich nourishment. They point out that their results do away with the boundary line between the genera *Euglena* and *Astasia* which have been separated on the basis of color (chlorophyll and stigma). Color, therefore, is not a sound character on which to base a classification of *Euglena*-like forms.

R. P. Hall and Jahn (1929*a*) say "A bifurcation of the flagellum is characteristic of the different species of uniflagellate Euglenidae examined and the flagellum in such species always shows a 'flagellar swelling' at the level of the stigma (which is always present) in vegetative stages. . . . Such structural features, however, have not been observed in the vegetative stages of any of the non-chlorophyll-bearing species examined by us or by other workers."

Further, they believe that certain stigma-bearing but chlorophyll-free flagellates, described in the literature as *Astasia*, should be put in the genus *Euglena*. They made no observations upon the conditions of the flagellum in these forms.

Bělař (1916) was of the opinion that *Astasia captiva* was an *Euglena*. He possibly was influenced by the presence of a stigma in this form, even though Beauchamp states that it was a colorless rudiment.

Because of the presence of a definite stigma and the bifurcation of the flagellum, the writer is of the opinion that this new form should be placed in the family Euglenidae Stein. If it is placed in this family, obviously it belongs in the genus *Euglena* Ehrbg. because of its morphological features, which would preclude its belonging to any other genus of this family.

SUMMARY

A new species of chlorophyll-free *Euglena*, *E. leucops*, is described, and though it lacks color, the presence of a stigma and the bifurcation of the flagellum place it in the family Euglenidae.

A flagellum is not present while the parasite is in the host but is elaborated quickly outside the host if favorable conditions of osmotic pressure prevail. This organelle is capable of repeated growth.

The stigma has been followed through binary fission and found to divide, but not always equally.

Nuclear division is described. The "chromosomes," which remain distinct, even in the resting nucleus, divide transversely during the metaphase.

Osmotic pressure apparently may initiate and accelerate cell division.

Attempts to cultivate the parasite *in vitro* failed.

The host may be easily cultivated and the infection readily continued.

No evidence of encystation has been observed.

Observations upon the flagellates for more than a year have given no evidence of a method of division other than binary fission involving mitosis.

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ALTERNATION OF GENERATIONS IN THE ROTIFER *LECANE INERMIS* BRYCE

I. Life Histories of the Sexual and Non-sexual Generations

HELEN MAR MILLER[1]

(From the Zoological Laboratory of the Johns Hopkins University)

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The Organism

The small loricate rotifer, *Lecane* (*Distyla*) *inermis* Bryce, presents many advantages for the study of the alternation of parthenogenetic and bisexual generations, common among the rotifers, and of the life histories of the diverse types of individuals that occur. It is hardy, easily cultivated, multiplies rapidly and changes readily from the non-sexual to the sexual condition and vice versa. The entire life of the female lasts usually but nine or ten days, and the first offspring appear on the second or third day. This organism is of interest as a representative of a genus in which the occurrence of males has not

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hitherto been described (Wesenberg-Lund, 1930, p. 94). The male of this species was first observed by Finesinger subsequent to his study (1926) of the inheritance of certain environmental effects in a succession of generations of parthenogenetic females.

Lecane inermis is one of the slow-moving rotifers, common in wet sphagnum. It belongs to the family Euchlanidæ, which is characterized by the possession of a lorica composed of two parts, a dorsal and a ventral plate, connected by lateral sulci. In *Lecane inermis* the lorica is extremely flexible, so that dorsal and ventral plates are barely distinguishable; the animal therefore resembles somewhat the more primitive, soft-bodied notommatids, with which the euchlanids are classified by Wesenberg-Lund (1929). A full description of this and other species of *Lecane* is given by Harring and Myers (1926). The individuals (females) of the stock employed in the present investigation are somewhat larger than the dimensions given for this species by Harring and Myers. Adults are 175–190 μ in length by 65–75 μ in breadth; newly-hatched individuals are about 130 μ in length by 33 μ in breadth.

As is well known, the rotifers of most common occurrence are the parthenogenetic or amictic females, and specific descriptions are based, in large measure, on these. In a single species there may occur, however, three sorts of individuals: the common amictic or non-sexual females, the mictic or sexual females, and the males. An outline of the change of generations will facilitate the understanding of the present study.

Outline of the Change of Generations

(Compare Fig. 1)

The common non-sexual or amictic females (*A*) multiply exclusively by parthenogenesis. They deposit eggs (*f*) that are ellipsoid in form, with a thin, smooth, outer membrane. These eggs carry the diploid number of chromosomes, are incapable of fertilization and produce females, usually like the mother, so that multiplication by (diploid) parthenogenesis may continue for many generations. But among the females produced by such eggs are at times individuals (*M*) that bring forth small eggs (*m*) which develop into the small, male individuals. These females are called mictic because their eggs are capable of being fertilized. They resemble outwardly the amictic females. The small, male-producing eggs carry the haploid number of chromosomes. There is some evidence that the male embryo may either double its chromosomes during development (*Asplanchna intermedia*, Tauson, 1924) or develop entirely with the haploid number (*Asplanchna amphora*, Whitney, 1929). When the mictic females are fertilized they produce, instead of the small, haploid, male-producing eggs, larger, dark eggs with a thick, horny shell. Such an egg has, of course, the diploid number of chromosomes. It remains unhatched for some time, and then produces a parthenogenetic or amictic female, with the diploid number of chromosomes. From such a female, commonly spoken of as the stem mother, begins again the cycle of diverse generations just described.

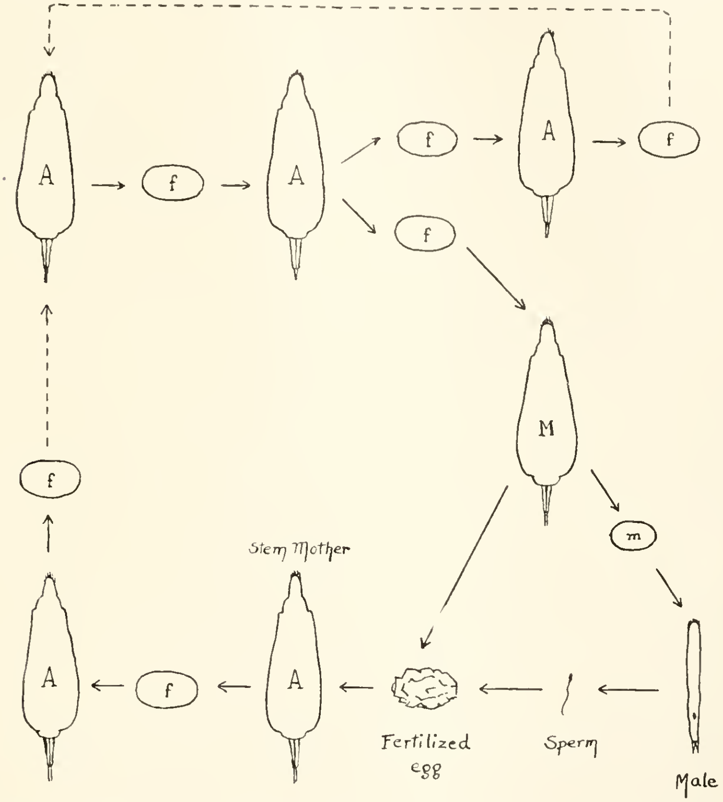


FIG. 1. Diagram showing the alternation of sexual and non-sexual generations in a bisexual rotifer, such as *Lecane inermis*. The most common type of individual, the non-sexual "amictic" female (*A*), reproduces exclusively by diploid parthenogenesis. Its eggs (*f*) develop invariably into females, which are usually amictic, so that multiplication by diploid parthenogenesis may continue for many generations (as indicated by the long, broken-lined arrows). But the daughters of the amictic female may be "mictic" (*M*). Their eggs are haploid, and develop parthenogenetically (*m*) into males, or, if fertilized, produce non-sexual amictic females, the "stem mothers," with which the cycle begins anew.

The purpose of the present investigation is to analyse this cycle in the species *Lecane inermis;* to give an account of the different types of individuals composing it, and to discover the factors that cause their production. This first paper presents a description of the different types of individuals, with a comparative study of their life histories. A later paper, now in preparation, will deal with the factors causing the change of generations.

Culture Methods

Lecane inermis is readily cultivated in grain infusions of proper strength, in malted milk, and in some standard inorganic culture media to which are added suitable food organisms. Oat infusion, used by Jennings and Lynch (1928—I) for *Proales sordida*, is (as they observe) a satisfactory medium also for *Lecane inermis*. An infusion of suitable strength is prepared by boiling twenty flakes of rolled oats for three minutes in 100 cc. of spring water, filtering while hot, and allowing to stand 24 hours before using. This "standard" oat infusion was employed in the comparative studies of the life histories of the different types of individuals and in the early part of the experimental work.

For precise experimental study of the influence of the environment on the kinds of individuals that occur, a more exactly controllable culture medium is necessary. Luntz (1926, 1929) has demonstrated the usefulness of synthetic inorganic media, such as the Benecke and Knop solutions, in the experimental investigation of the life cycle of rotifers. The food is controlled by the use of pure strains of food organisms, and the pH and initial salt concentration are likewise under control.

Benecke's solution in 0.07 per cent concentration, with addition of suitable food organisms, provides a most satisfactory culture medium for *Lecane inermis*. To it were added pure strains of certain bacteria, *B. proteus*, *B. subtilis* and *B. coli* (obtained from the School of Hygiene of the Johns Hopkins University). Such pure strains are inadequate as food, the rotifers surviving in them for but three generations. A mixture of two or three of these strains of bacteria is more satisfactory. Addition of green algæ to the bacteria gives still better results. A number of different algæ, obtained from the laboratory of Pringsheim at Prague, were tested. Species of *Chlamydomonas* and *Euglena* were apparently too large to serve as food for *Lecane inermis*. *Chlorella vulgaris* Beyerinck proved to be suitable, particularly when combined with *B. proteus*. The mixture of *Chlorella* and *B. proteus* has been employed in much of the experimental work. *Chlorella* is grown on beef agar, *B. proteus* on bacteriological agar.

A fresh suspension of these two food organisms in the culture solution is prepared daily immediately before the transfer of the rotifers.

In order to avoid contamination with other organisms, bacteriological precautions are observed in the preparation of the culture medium and food suspension, and in the handling of the rotifers. The method is, with a few necessary alterations, that devised by Raffel (1930) for the culture of *Paramecium*. The rotifers are cultivated individually on hollow-ground slides, placed in sterile Petri dishes containing distilled water. They are transferred daily with sterile, cotton-stoppered pipettes. A fuller account of the method of culture will be given in a later paper on the factors causing the change of generations.

Life Histories of the Diverse Types of Individuals

Knowledge of the general biology of the bisexual generations of rotifers is not extensive. This is doubtless due in part to the rare occurrence of sexual individuals in many of the species that have been employed for laboratory investigations. There have been few detailed comparative studies of the lives and activities of the mictic and amictic females, though such studies are much needed for an understanding of the fundamental difference between them, and of the different rôles they play in the life history of the species. Knowledge of the life history of the males is in little better case. *Lecane inermis* is a favorable organism for supplying these needs, since it readily yields an abundance of all types of individuals.

The Amictic and the Unfertilized Mictic Females: Comparison

The amictic and mictic females of *Lecane inermis* resemble each other closely, but differ markedly in certain features of their life histories, particularly in fecundity and length of life.

The mature females, whether amictic or mictic, present the appearance shown in Fig. 2.

As in *Proales sordida* (Jennings and Lynch, 1928—I), four periods may be distinguished in the life of the individual: (1) The period of embryonic development, from the deposition of the egg to hatching; this requires, in *Lecane inermis*, about one and one-half days. (2) The immature period, one of rapid growth and activity, concluding as a rule on the third day with the deposition of the first egg. (3) The period of fecundity, five or six days during which the eggs one at a time are matured and deposited in regular succession. (4) The post-fecund period, or period of old age, during which the activities of the female gradually cease, structural degeneration sets in and death ensues, usually on the ninth day, though it may be deferred for varying periods, depending on the conditions.

The amictic female.—The term *amictic* was first applied by Storch (1924) to the commonest type of rotifer female, since it cannot reproduce by amphimixis. The cytological investigations by Whitney, Storch, Nachtwey and others, of several species of rotifers, have shown that the eggs of the amictic female invariably develop by diploid parthenogenesis and produce diploid females, which may be either amictic or mictic.

The amictic females of *Lecane inermis* produce eggs that are ellipsoid in form and measure about 64 μ by 38 μ (Fig. 6). They are covered with a thin membrane surrounded by a gelatinous layer which swells, separates from the egg and disappears before the embryo emerges, about 30 hours after deposition.

The mictic female is capable of reproduction by amphimixis. Its eggs, as shown by the above-mentioned investigators, undergo reduction in the number of chromosomes. The mictic female of *Lecane inermis*, although indistinguishable in appearance from the amictic female, is immediately recognizable by the kinds of eggs it produces.

EXPLANATION OF PLATE

These drawings were reconstructed from many camera lucida sketches, for which a Zeiss microscope was used with oc.15x, obj.8.3, and with oc.15x, (Leitz) obj.3. The magnification of the seven figures is 518 diameters; this magnification was obtained by doubling the average dimensions of sketches of eggs and of living individuals, made with oc.15x, (Leitz) obj.3.

Abbreviations

c, contractile vacuole
d, rudiment of anterior portion of the digestive system
g, gastric glands
ex, excretory (?) granules
i, intestine
m, mastax
p.g., prostate (?) glands
o, ovary
oe, œsophagus
r, retro-cerebral organ
t, toes
te, testis
v.d., vas deferens

FIG. 2. Mature female (mictic or amictic); dorsal view.

FIG. 3. Young female, shortly after hatching; lateral view, showing prominent mastax, gastric glands, œsophagus, intestine and contractile vacuole.

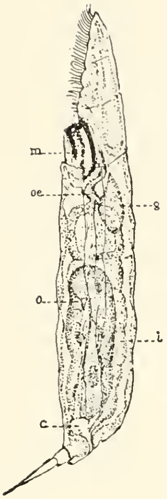
FIG. 4. Male; dorsal view.

FIG. 5. Male; lateral view, for comparison especially with Fig. 3. The digestive system is represented only by an anterior rudiment; the contractile vacuole is absent.

FIG. 6. The (female-producing) egg of the amictic female, several hours after deposition; lateral view. The gelatinous layer has separated from the egg membrane.

FIG. 7. The (male-producing) egg of the mictic female, before the initiation of cleavage.

FIG. 8. The fertilized (female-producing) egg of the mictic female.



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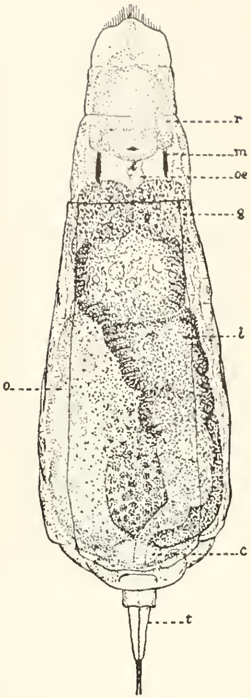

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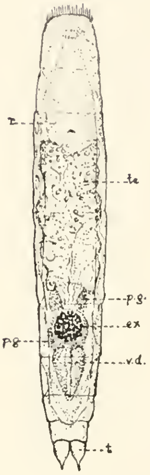
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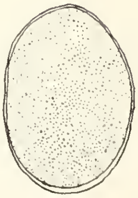

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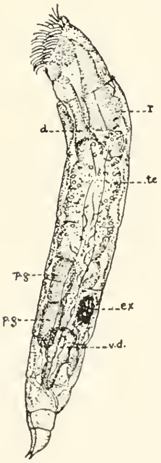
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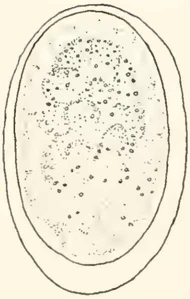

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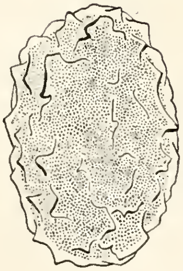

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If unfertilized, the eggs (Fig. 7) resemble the female parthenogenetic eggs, except that they are smaller (44 μ by 32 μ) and produce males.

The fertilized eggs (Fig. 8) are larger (64 μ by 38 μ), covered with a thick, horny shell, and produce amictic females. They are very black when laid, but the color fades to light brown in the course of several days. In this condition they remain for a period varying from a few days to two months. They withstand drying, which does not seem to alter their hatchability. Ordinarily, only 20 per cent to 30 per cent hatch in spring water or in oat infusion. Further investigation of the hatchability of the fertilized eggs is much needed. The course of development of these eggs has not been studied in detail. Apparently, cleavage begins shortly after deposition, but the greater part of embryonic development occurs during the 24 or 48 hours prior to hatching. The embryo emerges through an irregular break in the side of the shell.

The life histories of 108 amictic and 111 unfertilized mictic females were studied and compared in detail. These individuals were characterized by a high degree of genetic uniformity, since they were derived recently by parthenogenesis from a single common ancestor, and since they were derived from young mothers, less than 4 days old. This latter precaution eliminated the possibility of certain intrinsic differences, in fecundity, embryonic mortality and in the duration of certain periods in the life history, which result, in *Proales sordida* (Jennings and Lynch 1928–I, II), from differences in the ages of the parents.

The experimental females were cultivated individually under similar conditions, in small groups, during a period of two months. Uniformity of cultural conditions was obtained as follows. Small mass cultures, of 15 to 25 individuals in three drops of oat infusion, were maintained, the individuals being transferred daily to new fluid. Female-producing parthenogenetic eggs were removed from these cultures at the time of daily transfer, and isolated in depression slides, each one in three drops of oat infusion. The eggs isolated at any one time had thus been produced during the preceding 24 hours. The records of ages begin at the time of isolation, so that they are accurate only to a period of within 24 hours.

The eggs hatched as a rule during the first day after isolation. The young females grew rapidly and began to deposit eggs on the second day. Not until then could the individuals be recognized as mictic or amictic. They were transferred daily to clean slides and twenty-four hour infusion, at which time the number of eggs produced and other details were recorded for each mictic and amictic female.

TABLE I

Lecane inermis. Records of the numbers of eggs produced daily, throughout life, and of the total life duration, for typical contemporaneous amictic and unfertilized mictic females. *n. h.* signifies not hatched (on the first day); *d* signifies that the individual died on the day so marked.

Successive days of life		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total number of eggs	Length of life in days
Number of eggs laid by five amictic females	1	0	3	4	6	4	2	0d										19	7
	2	0	2	5	6	4	3	1d										21	7
	3	n.h.	1	4	5	6	(lost)												
	4	0	3	4	5	3	4	0d										19	7
	5	0	4	5	5	4	2	1	0d									21	8
Number of eggs laid by seven unfertilized mictic females	1	n.h.	1	4	4	4	3	0	0	0	0	0	0	0	0	0	0d	16	16
	2	0	3	4	4	3	0	0	0	0	0	0d						14	11
	3	0	3	4	5	3	0	0d										15	7
	4	0	3	4	4	4	1	0	0	0	0d							16	10
	5	0	2	3	4	4	3	0	0	0	0	0d						16	11
	6	1	4	4	3	2	0	0	0	0d								14	9
	7	1	4	4	4	3	0	0	0	0	0d							16	10

Since the male embryos require 30 hours or more for development, fertilization of the mothers by the sons was impossible, so that the mictic females as well as the amictic females reproduced throughout life only parthenogenetically.

During the two months of cultivation, the temperature varied from 19° to 24° C. Daily variations did not exceed one and one-half degrees. Although the mictic and amictic individuals were not distributed throughout this period in equal numbers, certain striking differences are observable in the life histories of the two kinds of females that cannot be attributed to differences in temperature, since they are exhibited by contemporaneous individuals subjected as nearly as possible to the same conditions of culture. Large contemporaneous cultures of the two types of females were at the time impracticable.

Table I gives the life histories with relation to the number of eggs produced daily, and the length of life, for two small groups of contemporaneous females of the two types. From Table I it is at once apparent that the mictic females (unfertilized) produce fewer eggs than the amictic females, but usually live longer. Further, on the whole, the egg-laying period of the mictic females terminates earlier.

Length of life and distribution of mortality

Table II gives for the individuals of the amictic and mictic populations the length of life in days (after their isolation as eggs); also the biometric constants, and the proportions that lived for the modal number of days, less than the mode and more than the mode.

As Table II shows, the largest number of individuals died, in both populations, on the ninth day, so that this is the modal length of life for both sets. But many of the mictic females (55.9 per cent) lived beyond this age, while fewer of the amictic females (32.4 per cent) did so. The maximum age reached by the amictic females was 15 days, while 13 per cent of the mictic females lived beyond this age, two reaching the ages of 27 and 28 days respectively. The mean length of life of the amictic females is 8.9 ± 0.11 days; of the mictic females, 11.0 ± 0.28 days. There are thus very characteristic and significant differences in the length of life reached by the amictic and mictic females respectively. Further, the duration of life is much more variable for the mictic than for the amictic females. For the amictic females the standard deviation in length of life is 1.72 ± 0.08 days, and the coefficient of variation is 19.28 ± 0.92 per cent, while for the mictic females the standard deviation is 4.37 ± 0.20 and the coefficient of variation 39.41 ± 2.04 per cent.

These differences in length of life are very noticeable in observation

TABLE II

Lecane inermis. Length of life in days (after their isolation as eggs) of amictic and unfertilized mictic females. The figures given in the body of the table are the numbers of individuals of each population that reach the ages in days indicated in the upper line of the table.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Total
Number Amictic Females..................						7	20	15	31	16	12	5	1	0	1														108
Number Mictic Females..................					1	3	11	14	20	16	13	8	7	1	3	2	1	1	2	1	2	1	1	1	0	0	1	1	111

	Mean	Standard Deviation	Coefficient of Variability	Mode	Per cent below mode	Per cent at mode	Per cent above mode
Amictic Females..................	8.90 ± 0.11	1.72 ± 0.08	19.28 ± 0.92	9	38.88	28.70	32.41
Mictic Females..................	11.10 ± 0.28	4.37 ± 0.20	39.41 ± 2.04	9	26.13	18.02	55.86
Difference..................	− 2.20 ± 0.30	− 2.65 ± 0.21	− 20.13 ± 2.24	0	12.75	10.68	− 23.45

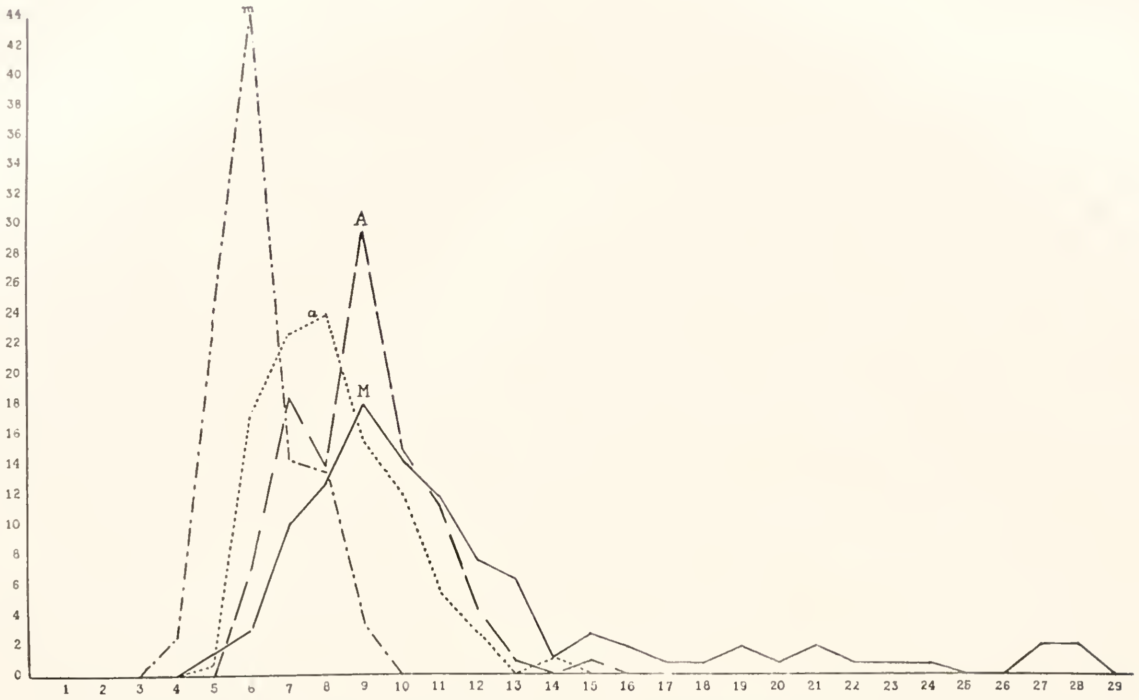

FIG. 9. *Lecane inermis*. Graphs showing the proportions of individuals reaching the different ages, in the amictic population (*A*), and in the population of unfertilized mictic females (*M*); also the ages at which the amictic females (*a*) and the unfertilized mictic females (*m*) cease egg-deposition. Age reckoned in days from time of isolation (within 24 hours after deposition). Horizontal scale, days of life; vertical scale, percentages of each population.

of the living animals. The mictic females cease egg-laying early in life, but most of the individuals continue to swim about actively for several days after, a few of them for many days. In these long-lived individuals physical decline is very gradual. The gastric glands and mastax become blackened and locomotion wanes, typical signs of structural degeneration. But the mictic females may linger on for many days in this condition. The amictic females, on the other hand, frequently show signs of age before the last egg is produced and all are dead within two days thereafter.

The distribution of mortality is represented graphically in Figs. 9 and 10. In Fig. 9, curves *M* and *A* show the proportions of the mictic and amictic populations respectively that reached the different ages. Figure 10 represents mictic and amictic individuals as though contemporaneous, and shows the proportions of each population present at the beginning of each day. Embryonic mortality is negligible; only one egg of the 220 eggs isolated failed to hatch. In this respect early-born mictic and amictic females resemble early-born populations of *Proales sordida* (Jennings and Lynch, 1928—II, Fig. 15). None die during immaturity; all live through the fourth day. All of the amictics and 99 per cent of the mictics survive the fifth day after isolation, that is, four days of fecundity. On every day thereafter, fewer amictic than mictic females are still alive. In both populations the mortality rate is low on the sixth day, higher on the next and reaches a maximum on the ninth day. It thereafter decreases until all the amictics are dead on the fifteenth day and the mictics linger on, two or three dying on successive days until the twenty-eighth day.

To what extent is the difference in longevity of the mictic and amictic females correlated with, or the result of, difference in fecundity? Information regarding this question is obtained from examination of the distribution of fecundity in the two populations, the rate of egg-production, the duration of the post-fecund period and the age at which the production of eggs ceases.

Fecundity

Inspection of Table I, giving the life history records for contemporaneous mictic and amictic females, has shown that the mictic female deposits only 14 to 16 of the small, male-producing eggs; the amictic female deposits 19 to 21 of the larger, female-producing eggs. Table III presents a comparison of the distribution of fecundity in the two populations, with the biometric constants; and Fig. 11 shows the proportions of individuals that produced the different numbers of eggs. The differences in the fecundity of the two types of female are striking. The amictic females produce 13 to 24 eggs per individual; the modal

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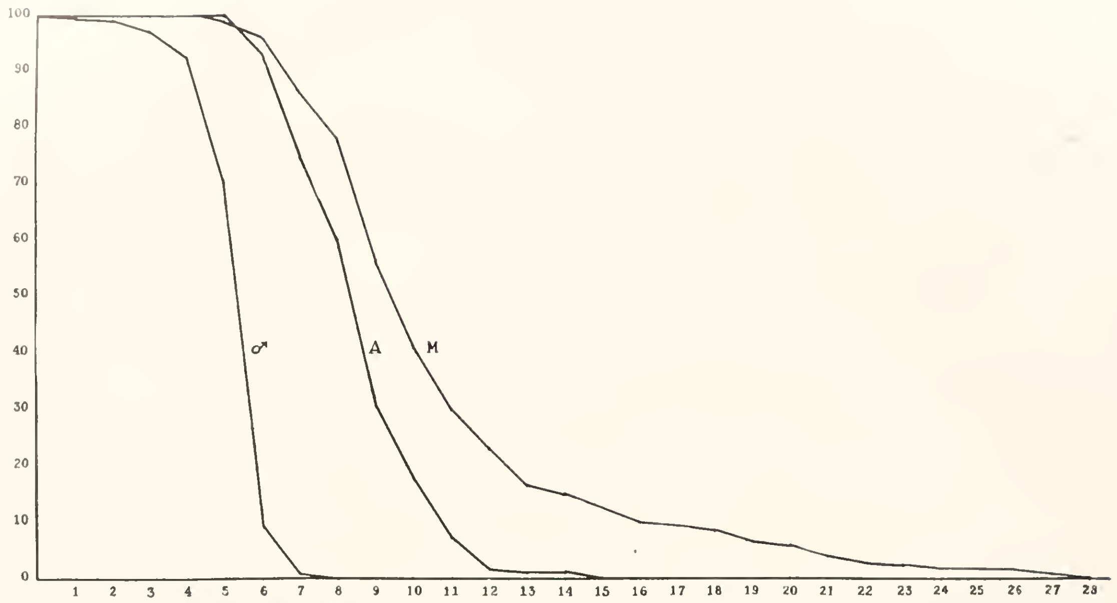

FIG. 10. *Lecane inermis.* Mortality and survivorship diagrams for the three different types of individuals, the amictic females (*A*), the unfertilized mictic females (*M*) and the males (♂). Horizontal scale, days of life; vertical scale, percentages of the populations surviving on the successive days.

TABLE III

Lecane inermis. Comparison of the fecundity of amictic and unfertilized mictic females. Total number of eggs produced during its life-time by each of the 111 mictic and 108 amictic females.

Number of eggs	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Total
Number amictic females..........														1	0	1	3	3	5	11	18	27	17	16	6	108
Number mictic females...........			1	0	0	0	0	0	0	0	0	2	10	15	29	31	23									111

	Mean	Standard Deviation	Coefficient of Variability
(*a*) Amictic Females (108)..	20.73 ± 0.13	2.06 ± 0.09	9.92 ± 0.46
(*b*) Mictic Females (111)..	14.22 ± 0.11	1.74 ± 0.08	12.22 ± 0.56
(*c*) Mictic Females, without individual that produced 2 eggs (110)....	14.33 ± 0.08	1.29 ± 0.06	9.03 ± 0.41
Differences *a–b*..	6.51 ± 0.17	0.32 ± 0.12	– 2.30 ± 0.73
a–c..	6.40 ± 0.16	0.77 ± 0.11	0.89 ± 0.62

fecundity is 21 and the mean 20.7 ± 0.13. The mictic females produce 11 to 16 eggs (with the exception of one individual which produced only two eggs and died on the fifth day); the modal fecundity is 15, and the mean 14.2 ± 0.11. Thus, the mictic females produce regularly only two-thirds as many parthenogenetic eggs as the amictic females. The fact that the coefficients of variability for both populations are low, 9.9 per cent for the amictics and 12.2 per cent for the mictics, emphasizes the difference in the fecundity of the two types of females.

Excluding the single exceptional mictic female which produced only two eggs, the mean fecundity for the mictic population (110 individuals) is 14.3 ± 0.08, the standard deviation 1.29 ± 0.06, which is less than that of the amictic population by 0.77 ± 0.11, a significant difference, and the coefficient of variability is reduced to 9.03 ± 0.41, which is almost the same as that of the amictic females.

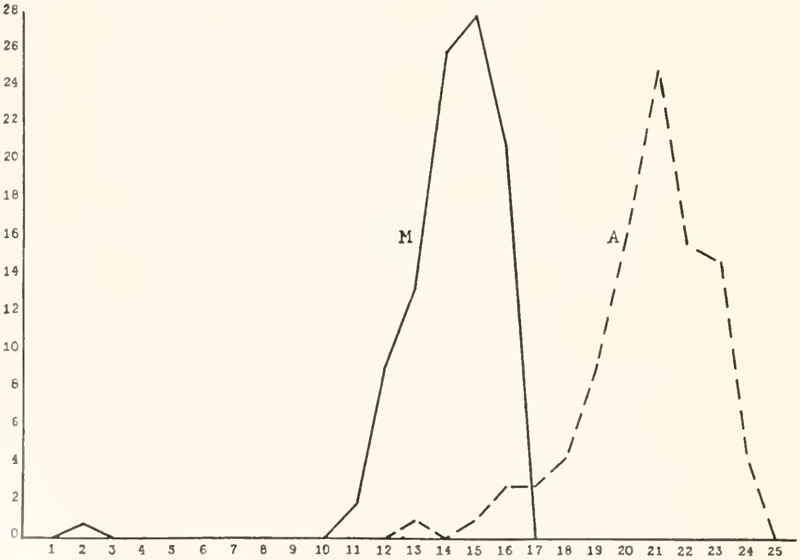

FIG. 11. *Lecane inermis.* Graphs showing the numbers of eggs produced by individuals of the two populations, the amictic females (*A*) and the unfertilized mictic females (*M*). Horizontal scale, numbers of eggs produced; vertical scale, percentages of the populations producing the different numbers of eggs.

The two populations differ further in the fact that a very much greater proportion of the mictic females produced the maximum or nearly the maximum number of eggs. This is clearly observable in Fig. 11. Whereas 48 per cent of the mictic females produced 15 or 16 eggs, only 21 per cent of the amictics produced 23 or 24 eggs. The mode and the mean fecundity more nearly coincide with the maximum in the mictic population.

Duration of the fecund period and rate of egg-production

It will be recalled that the mictic and amictic females mature and deposit the first eggs at about the same time, during the second day after isolation, also that the mictic female deposits but two-thirds as many eggs as the amictic female. If the male-producing parthenogenetic eggs are deposited at the same rate as the female-producing eggs, the fecund period of the mictic female should be only two-thirds as long as that of the amictic female.

Table IV

Lecane inermis. Duration of the period of fecundity of amictic and unfertilized mictic females. The figures given in the body of the table indicate the number of individuals in each population that required for the production of their eggs the number of days indicated on the upper line.

Number of days	2	3	4	5	6	7	8	9	10	11	Total
Number of Amictic Females..........			4	30	30	19	14	9	1	1	108
Number of Mictic Females...........	1	0	22	57	26	5					111

	Mean	Mode	Standard Deviation	Coefficient of Variability
(*a*) Amictic Females (108)....	6.42 ± 0.09	5, 6	1.44 ± 0.07	22.45 ± 1.08
(*b*) Mictic Females (111)......	5.10 ± 0.05	5	0.83 ± 0.04	16.22 ± 0.75
(*c*) Mictic Females, without individual that produced 2 eggs and died on 5th day (110)....................	5.13 ± 0.05		0.78 ± 0.04	15.13 ± 0.70
Differences *a–b*.............	1.32 ± 0.11		0.61 ± 0.08	6.23 ± 1.32
a–c.............	1.29 ± 0.10		0.66 ± 0.08	7.32 ± 1.29

In Table IV is given the number of days during which the females of both classes continue to produce eggs. The minimum number of days of the fecund period was the same for both (with the exception of the one individual, which produced only two eggs and died on the fifth day); but the total number of days ranged from 4 to 7 for the mictic females, from 4 to 11 for the amictic females. The mode for the mictics is five days; for the amictics 5 and 6 days are equally common. For 23 per cent of the amictics the period of fecundity exceeds the maximum period for the mictics. The mean for the mictics is 5.10 ± 0.05 days, for the amictics 6.42 ± 0.09 days. The standard deviation is, for the amictics 1.44 ± 0.07, for the mictics 0.83 ± 0.04. The coefficient of variability is, for the amictics 22.45

± 1.08, for the mictics, 16.22 ± 0.75. The differences in the mean, standard deviation and the coefficient of variability of the two populations are statistically significant, regardless of whether the one exceptional mictic female is included in the calculations (Table IV).

The mictic female requires on the average only one and three-tenths days less to produce its eggs, though these are fewer by one-third than the number produced by the amictic female. This means that, although both types of females produce 3 to 5 eggs per day, rarely 6, the mictic female deposits its small (male-producing) eggs, on the average, in less rapid succession than the amictic female deposits its larger (female-producing) eggs. The mictic female deposits, on the average, 3.8 eggs per day, or one every 8.6 hours; the amictic female 3.2 eggs per day, or one every 7.5 hours. These figures represent the quotients of the mean fecundity divided by the mean duration of the fecund period. Additional data regarding the rate of egg-deposition are given later in the paper.

Age at cessation of fecund period and duration of post-fecund period

In Table V is given the duration of the post-fecund period for all individuals of the two populations. Most of the amictic females (59 per cent) died within 24 to 36 hours after deposition of the last egg; 33 per cent died within one day, all died within two days thereafter.

TABLE V

Lecane inermis. Duration of the post-fecund period of amictic and unfertilized mictic females. Those individuals that produced eggs on the day on which they died, or as many as four eggs on the day preceding death are considered as having a post-fecund period of one day or less; if they produced no eggs on the last day, but one to three on the preceding day, they are listed as having a post-fecund period of one to one and one-half days; if they produced no eggs on the last two, three or four days, their post-fecund period is correspondingly two, three or four days, etc.

Number of Days	One day or less	1–1½	2	3	4	5	6	7	8	9	10
Number of Amictic Females.........	36	64	8								
Number of Mictic Females..........	2	15	20	18	17	6	11	2	2	3	3

Number of Days	11	12	13	14	15	16	17	18	19	20	21	22	Total
Number of Amictic Females...													108
Number of Mictic Females....	1	1	3	1	2	0	0	2	0	0	1	1	111

Not so with the mictic females. Only 33 per cent of the entire population died within two days after the cessation of egg-deposition.

Of these, 20 individuals survived for two days, 15 for one and one-half days, only two died within 24 hours after deposition of the last egg. While two days after the cessation of egg-production is the commonest period of death, three or four days are almost equally common; five and six days less frequent. A few individuals survive 7 to 22 days after the cessation of egg-production, there being, however, but one, two or three individuals in each class. Yet 20 per cent of the mictic females lived more than 6 days after the fecund period had ended.

As set forth in the preceding section, the mictic females cease to deposit eggs earlier, on the average, than the amictic females. The post-fecund period of the mictic females is thus extended in this way by about 1.3 days. However, this accounts for only a small fraction of the greater length of life of the mictic females after the cessation of egg-deposition. The mictic females have, on the whole, a much greater ability to survive the period of fecundity than the amictics.

This striking difference is shown for the entire populations in Fig. 9, representing the distribution of ages at the cessation of egg-production in comparison with the distribution of ages at death.

TABLE VI

Lecane inermis. Comparison of the ages at which amictic and unfertilized mictic females cease the deposition of eggs.

Age in Days	4	5	6	7	8	9	10	11	12	13	14	Total
Number of Amictic Females......	0	1	19	25	26	17	13	6	0	0	1	108
Number of Mictic Females........	3	24	49	16	15	4						111

Table VI gives for both populations the number of individuals that cease egg-production at the different ages. The fecund period of the amictic females terminates usually on the seventh and eighth days; that of the mictic females usually on the sixth day. As stated in a previous section, all of the amictic females and all but one mictic female live through four days of egg-production. Mortality then begins in both populations and rises to a maximum on the ninth day. Thus, the maximum mortality in the amictic population occurs from one to two days after the maximum number of individuals cease egg-production; in the mictic population, three or four days thereafter. After the ninth day, the distribution of mortality of the amictic females follows the cessation of egg-production within one or two days. The mictic females, however, have all ceased egg-production at the end of the ninth day, and 56 per cent are still alive. These die at a slow rate until the 14th day, the remaining 15 per cent at a fairly uniform rate until the 28th day.

Relation of length of life to fecundity and rate of egg-production

The foregoing data indicate that the difference in the length of life of mictic and amictic females results, in part, from the difference in their ability to survive the fecund period. This has been evidenced by the relative duration, in the two populations, of the fecund and post-fecund periods, and the relative ages at which egg-production ceases.

We may inquire further into the extent of the relationship between length of life and fecundity of mictic and amictic females, by comparing the fecundity, rate of egg-production, and the duration of the post-fecund period, in relatively short-lived, average-lived and long-lived individuals of each population. Those that lived less than 9 days, the mode, are classified arbitrarily as short-lived, those that lived 9 days as average-lived and those that lived more than 9 days as long-lived. The results of this comparison are presented in Tables VII to IX.

TABLE VII

Lecane inermis. Comparison of the relation of life duration to fecundity displayed by amictic and unfertilized mictic females. Individuals that lived less than 9 days, the mode, are called short-lived; those that lived 9 days are called average-lived, and those that lived more than 9 days, long-lived.

		Short-lived	Average-lived	Long-lived		Total
				Total	Those living beyond 15 days	
Amictic Females	Number	42	31	35	0	108
	Mean eggs	19.95	20.45	21.91		
Mictic Females	Number	29	20	62	14	111
	Mean eggs	14.0	14.05	14.2	14.64	

Table VII gives the mean fecundity for these groups. The short-lived amictic females produced, on the average, 19.95 eggs per individual, the average-lived 20.45, the long-lived 21.91. The difference in the mean fecundity of short-lived and long-lived amictic females is 1.96 eggs. The short-lived mictic females produced, on the average, 14.0 eggs per individual, the average-lived 14.05, the long-lived 14.2. The difference in the mean fecundity of short-lived and long-lived mictic females is 0.2 eggs, which is only one tenth of the difference (1.96) found in the corresponding groups of amictic females. The mean fecundity of those mictic females that lived beyond 15 days, and thus survived all the amictic females, is 14.6 eggs; that is, it is greater than the mean fecundity of short-lived mictic females by only 0.6 eggs. The positive correlation between length of life and the number of eggs produced is appreciably more marked for the amictic females.

TABLE VIII

Lecane inermis. Comparison of the relation of life duration to rate of egg-deposition (number of eggs per day) for amictic and unfertilized mictic females. The "mean rate" of egg-deposition is the average of the rates at which all the members of a particular group deposited their eggs. The rate of egg-deposition of each individual is computed by dividing the total number of eggs produced, by the number of days on which eggs were deposited by that individual.

		Short-lived	Average-lived	Long-lived		Total
				Total	Those living beyond 15 days	
Amictic Females	Number	42	31	35	0	108
	Mean rate	3.88	3.33	2.73		
Mictic Females	Number	29	20	62	14	111
	Mean rate	3.0	2.78	2.78	2.87	

The length of life of the amictic females bears a negative correlation with the rate at which they produce their eggs (Table VIII). The short-lived individuals are found to have deposited their eggs in more rapid succession than individuals that lived 9 days or longer. They deposited, on the average, 3.9 eggs per day, the average-lived 3.3 eggs per day, the longer-lived 2.7 eggs per day. The short-lived mictic females deposited 3 eggs per day, the average-lived 2.8, the long-lived also 2.8 eggs per day. Those in the latter group that lived 16 days or more deposited, on the average, 2.9 eggs per day. The length of life of the mictic females apparently bears little relation to the rate at which their eggs are deposited.

It should be noted also that each group of mictic females is characterized by an average daily egg-production almost the same (within 0.3 eggs) as the group of amictic females that produced their eggs most slowly, namely, the long-lived individuals. The amictic group with the highest rate of egg-deposition (the short-lived individuals) produce, on the average, 0.9 eggs per day more than the corresponding group of mictic females. These facts confirm and supplement data given in a previous section which indicate that the mictic female produces its eggs more slowly than the amictic female.

We find, furthermore (Table IX), in the amictic population, that whereas 25 (60 per cent) of the short-lived individuals died within 24

Table IX

Lecane inermis. Comparison of the relation of length of life to the duration of the post-fecund period for amictic and unfertilized mictic females. The figures in the body of the table indicate the number of individuals that survived after the cessation of egg-production for the number of days indicated on the upper line.

Number of Days		1 or less	1 to 1½	2	3	4	5	6	7	8	9–22	Total
Short-lived Females	Amictic	25	16	1								42
	Mictic	2	9	14	4							29
Average-lived Females	Amictic	6	22	3								31
	Mictic		5	2	8	5						20
Long-lived Females	Amictic	5	26	4								35
	Mictic		1	4	6	12	6	11	2	2	18	62
Total number	Amictic											108
	Mictic											111

hours after deposition of the last egg, only 6 (19 per cent) of the average-lived and 5 (14 per cent) of the long-lived died within this period. Twenty-two (71 per cent) of the average-lived and 26 (74 per cent) of the long-lived females survived for 24 to 36 hours after deposition of the last egg. Only 8 individuals (7 per cent of the entire population) survived as long as two days after the cessation of egg-production.

However, all of the mictic females, with the exception of two short-lived individuals, lived more than 24 hours after the cessation of egg-production. Most of the short-lived mictic females survived one and one-half or two days. The average-lived survived usually three days, though one and one-half and three days were almost equally common. The long-lived individuals, which constitute 56 per cent of the population, survived one and one-half to 22 days after the cessation of egg-production, commonly 4 to 6 days. Only 10 per cent survived less than 4 days; 20 per cent survived longer than 6 days.

In short, the amictic females that lived for less than 9 days produced their eggs, on the average, in more rapid succession than those that lived longer; they also produced fewer eggs and died usually within 24 hours after deposition of the last egg. These facts suggest that rapid rate of egg-production exhausts the short-lived amictic females, rendering them incapable of completing the transformation of their germ cells into eggs, thus reducing their fecundity. So strenuous is the fecund period that none is able to survive more than two days after its cessation.

In the mictic population, as in the amictic population, the longest-lived individuals are those that have, on the average, produced their eggs more slowly, have produced a larger number of eggs and have survived more than a day after the deposition of the last egg. However, the length of life of the mictic female is correlated in less degree than that of the amictic female with the number of eggs produced and the rate at which they are produced. The short-lived mictic females usually survive as long after the cessation of egg-deposition as the longest-lived amictic females; they very infrequently show signs of physical exhaustion before the completion of egg-production. We may conclude, then, that the greater longevity of the mictic females, which involves a greater ability to survive the fecund period, results, in part at least, from the fact that for them the process of egg-production is less strenuous. This is to be expected in view of the fact that they produce fewer, smaller eggs at a less rapid rate than the amictic females.

General and comparative

Under similar conditions of cultivation amictic and unfertilized mictic females of *Lecane inermis* differ markedly in length of life, fecundity, rate of egg-production, duration of the fecund and post-fecund periods and in the degree of correlation between fecundity and length of life. How are these differences between the two types of females produced?

In their investigation of the diversities that arise within a clone of (amictic) females of the parthenogenetic rotifer, *Proales sordida*, Jennings and Lynch (1928) concluded that differences in length of life, among those individuals that hatch, are not an expression of intrinsic diversity. They result, rather, from the accidents of life, the interaction of the rhythmic processes of digestion and reproduction, combined with disturbances in these processes imposed by the daily transfer of the individuals to clean slides.

In *Lecane inermis*, the difference in the length of life of the mictic and amictic female results largely from the difference in the severity of the process of egg-production, hence in their ability to survive the fecund period. Both mictic and amictic females lived most commonly nine days, but many more mictic than amictic females lived beyond this age, presumably because they produce fewer, smaller eggs than the amictic female, at a slower rate, and cease egg-deposition at an earlier age. Thus, in *Lecane inermis*, differences in length of life are probably to be considered of an intrinsic nature only in so far as they result from intrinsic differences in fecundity.

The mictic females of *Lecane inermis* resemble the females of *Proales sordida* in that some of them are able to live for many days after the cessation of egg-production. The amictic females of *Lecane inermis*, however, never survive more than 3 or 4 days thereafter. Both mictic and amictic females differ from *Proales sordida* in the degree of correlation between length of life and fecundity. For individuals of *Proales sordida* that live entirely through the period of fecundity (24 hours after deposition of the last egg) there is no correlation between length of life and the number of eggs produced; longer-lived individuals have, on the whole, produced neither fewer nor more offspring than the average (Jennings and Lynch, 1928—II, p. 371). In *Lecane inermis*, on the other hand, among those individuals that lived entirely through the fecund period, the longer-lived amictic females and those mictic females that lived for more than 15 days have a somewhat greater average fecundity than those that lived a shorter time.

What is the origin of the difference in fecundity which is, in *Lecane inermis*, largely responsible for the difference in longevity of the mictic and amictic female? In *Proales sordida*, intrinsic diversities in fecundity arise among the individuals of a clone in correlation with the size of the eggs from which they have hatched. Smaller (usually early-born) eggs produce less fecund individuals than larger (later-born) eggs. In order to eliminate diversities that might arise in this way, only early-born individuals were used in the present investigation. Although measurements have not been made, there is no observable difference in the size of the parthenogenetic eggs that produce the two different kinds of females of *Lecane inermis*. In the viviparous species *Asplanchna intermedia*, on the other hand, the mictic female is apparently derived from a smaller embryo and is less fecund than the amictic female (Tauson, 1925, p. 144).

As Jennings and Lynch point out (with respect to *Proales sordida*), differences in fecundity must proceed either from differences in the ability of the individuals to transform their germ cells into eggs under the conditions of cultivation employed, or from differences in the original number of their germ cells. There is (as they observe) evidence that the number of germ cells, as well as of the other cells of the body of rotifers, is fixed during the embryonic period. Cell mitoses are not observed later in life. Nachtwey (1925) has shown that the rotifer *Asplanchna priodonta* has usually 32 germ cells, derived by five cell cleavages from the single primitive germ cell. In view of this evidence, Jennings and Lynch (1928—I) have suggested that differences in the fecundity of the (amictic) females of *Proales sordida* that are derived from smaller eggs and those derived from larger eggs may result from differences in the original number of germ cells. Late-born individuals may have usually 32 germ cells, early-born individuals a smaller number, resulting from failure of a portion of the fourth cell generation to undergo another division.

In *Lecane inermis* the observed difference in the fecundity of the two types of female would result if the primitive germ cell of the mictic female undergoes regularly only four successive divisions, producing 16 oöcytes; and if, in the amictic female, one-half of the fourth cell generation regularly proceeds to a fifth cleavage, thus producing 24 germ cells. Cytological investigation would, of course, be required in order to determine whether there exists a difference in the original number of germ cells.

It is possible that, under conditions other than those of this investigation, the mictic female might be able to bring to maturity as many germ cells as the amictic female. It should be noted, however, that the differences in the fecundity and length of life of the two types of females have been observed during cultivation under widely different conditions; namely, in oat infusion and in Benecke solution at temperatures ranging from 18° to 24° C.

The observations of Whitney (1907) regarding the fecundity of mictic and amictic females of *Hydatina senta* are of interest here. At 20° to 22° C. both types of females produce about the same number of eggs; at 24°–26° C. the mictic female produces twice as many eggs as the amictic; at 26°–29° C. the mictic female produces four times as many eggs as the amictic female. Thus, in *Hydatina senta*, differences in the fecundity of the two types of female result observably from differences in response to the environment. The mictic female lives at all these temperatures slightly longer than the amictic female (Wesenberg-Lund, 1929). The relative longevity of the amictic and mictic female of *Hydatina senta* is not correlated with the relative fecundity, as in *Lecane inermis*.

For other species, information regarding the relative length of life, fecundity and rate of egg-production of the mictic and amictic female is not extensive.

The life history records of three mictic females of *Euchlanis triquetra* (Lehmensick, 1926) indicate that they produce the same number of eggs as the amictic female (24) and live about the same length of time, 21 days. But the mictic female produces its eggs more rapidly and thus lives longer after the cessation of egg-production.

The mictic female of *Asplanchna intermedia* (Tauson, 1925) produces fewer eggs, as mentioned above, and lives a shorter length of time than the amictic female. The mictic female matures on the first day, produces 10 to 12 eggs on the second day, which hatch on the third day; the mother dies after the hatching of the last male. The amictic female lives 5 or 6 days, produces daily no more than 4 eggs (probably 16 to 20 in all). The mictic female produces its eggs appreciably more rapidly.

In *Pterodina elliptica* (Luntz, 1926), the mictic female produces regularly six or seven eggs, the amictic female only five. There is no available information regarding the length of life. Physiological diversity is manifested by the inability of the mictic female to withstand certain conditions of osmotic pressure and pH in which the amictic female is perfectly normal.

Wesenberg-Lund states (1930, p. 31) that "if not fertilized, investigations hitherto carried out seem to show that the number of eggs laid by the two sorts of females is almost the same, but that those of the mictic female are laid in a much shorter time (Lehmensick, 1926, *Euchlanis triquetra*)."

We find, however, that the mictic females of some species, *e.g.*, *Asplanchna intermedia* and *Lecane inermis*, are regularly less fecund than the amictic females, whereas those of some other species, *e.g.*, *Pterodina elliptica* and *Hydatina senta* are, usually, more fecund. With regard to the relative rates at which the eggs are deposited by the two sorts of females, *Lecane inermis* is exceptional; the mictic female produces its eggs less rapidly than the amictic female.

Wesenberg-Lund (1930) presents a comparison of the mictic and amictic females on the basis of his extensive investigations carried out largely in nature, but supplemented by laboratory observations. The author stresses the physiological and biological differences between the mictic and amictic female and gives some additional data regarding their relative fecundity, rate of egg-production and length of life. In *Asplanchna sieboldi* the mictic females mature and begin to deposit eggs earlier than the amictic females. In isolation cultures they produced 14 to 16 eggs and lived 10 to 12 days. Amictic females, in mass cultures, lived only 8 days and produced 8 to 10 eggs. The author is inclined to believe (p. 156) that in nature the amictic females live longer than the mictics, especially at low temperatures. Concerning *A. brightwelli* he "conjectures that at the same temperatures the amictic females live some days longer than the mictic ones;

produce more young ones; and to a somewhat higher degree are able to accommodate duration of life and time of production of young ones to temperature" (p. 149). Regarding the rate of production of the parthenogenetic eggs, Wesenberg-Lund states (p. 210) that, in several species, "the mictic female is able to subdivide the yolk mass into small amounts, and produce about 12 eggs simultaneously, whereas the amictic females produce the eggs successively." Further evidence of physiological diversity is presented by certain parasitic species in which the amictic females are usually free-living, the mictic females always parasitic. Peculiarities in behavior exhibited by mictic females of some species after fertilization indicate that they are structurally as well as physiologically diverse.

In summary, the evidence at hand is sufficient to demonstrate that in different species the relative fecundity, length of life and rate of egg-production of the mictic and amictic female vary greatly. In every species which has been studied physiological differences between the two types of female are demonstrable; but investigations have not proceeded sufficiently far to admit of general conclusions regarding the nature of the fundamental diversity that distinguishes the mictic from the amictic female.

FERTILIZED MICTIC FEMALES

In the foregoing only unfertilized mictic females have been considered. The mictic female of *Lecane inermis*, like that of *Pterodina elliptica* and *Brachionus bakeri* (Luntz, 1926, 1929), and *Asplanchna sieboldi* (Wesenberg-Lund, 1930, p. 155), may be fertilized during its immature period or after having deposited parthenogenetic eggs. In some species the mictic female can be fertilized only when very young (*Euchlanis triquetra*, Lehmensick, 1926).

The mictic females of *Lecane inermis* resemble those of most species in that they may be fertilized by their own sons. The females of *Pterodina elliptica* are exceptional in that they are incapable of being fertilized by the male progeny of the same grandmother. In *Lecane inermis* cloacal copulation occurs. The toes of both male and female are bent ventrally, almost at right angles to the axis of the bodies, which are extended in opposite directions. The female continues to swim about, pulling the male along with her. Copulation lasts for at least five to ten minutes. The cloacal method of copulation is uncommon in rotifers, according to Wesenberg-Lund (1929). The males of most species attach themselves to the body of the female and in some manner pierce the body wall and deposit sperm directly in the body cavity.

As noted by all investigators of the bisexual rotifers, fertilization produces a visible effect upon the vitellarium. This organ becomes black during the growth of the fertilized egg. This, it is generally believed, results from the deposition of fat and the production of larger, darker yolk granules. In the course of 24 to 36 hours a fertilized egg matures and is deposited. The vitellarium is then quite colorless. The production of yolk and the deposition of a fertilized egg may be repeated four or five times.

Information regarding the fecundity and length of life of fertilized mictic females of this species is derived from the life history records of 18 individuals fertilized during immaturity and 5 individuals fertilized by their own sons. The individuals fertilized before maturity produced only fertilized eggs, the total number not exceeding five. They lived usually 4 or 5 days after the cessation of egg-production; the total length of life ranged from 10 to 25 days.

The life history records of the five females fertilized by their own sons at various times during maturity are given in Table X. These individuals produced 5 to 9 parthenogenetic eggs before being fertilized. They produced thereafter one to 4 fertilized eggs. They are typical in their ability to survive the fecund period. The total length of life ranged from 13 to 18 days.

Mictic females of this species have not been observed to deposit parthenogenetic eggs after once having produced fertilized eggs. This is not the case in *Hydatina senta* (Shull, 1910) and in several other species. Lehmensick (1926) suggested that fertilized mictic females return to the production of parthenogenetic eggs because all of their germ cells were not fertilized at the first mating, and Wesenberg-Lund has observed that after a second mating the production of fertilized eggs may be resumed (1930—II, p. 154). The failure of fertilized mictic females of *Lecane inermis* to produce parthenogenetic eggs probably indicates that more of their germ cells were fertilized than they were capable of bringing to maturity; for, frequently, after the cessation of egg-deposition the vitellaria contained dark yolk granules and fertilized eggs were present in various stages of growth.

Fertilization commonly reduces the fecundity of the mictic female. *Lecane inermis*, which produces, when fertilized, only 5 to 10 eggs rather than 16, is not exceptional in this respect. The length of life is, in this species, apparently not affected by fertilization.

THE MALES

The males of this species (Figs. 4 and 5) are strikingly different in appearance from the females, and, like most male rotifers, are to

TABLE X

Lecane inermis. Records of the numbers of male-producing and fertilized eggs produced daily, throughout life, and of the total life duration of five mictic females, fertilized at various times during the fecund period. The numbers given in the body of the table are the numbers of eggs deposited, on the days indicated on the upper line, by each of the five individuals. The designation (♂) below the numbers indicates male-producing eggs; numbers not so marked indicate fertilized eggs.

Individuals	Days of Life																		Fecundity			Life in days
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Male eggs	Fertilized eggs	Total	
1	0	5 ♂	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0d	5	2	7	18
2	0	0	2 ♂	3 ♂	2 ♂	0	1	1	1	1	0	0	0d						7	4	11	13
3	0	0	3 ♂	3 ♂	2 ♂	1 ♂	1	0	0	0	0	0	0	0	0	0	0	0d	9	1	10	18
4	0	1 ♂	3 ♂	3 ♂	1 ♂	1	1	0	0	0	0	0	0	0	0	0	0d		8	2	10	17
5	0	1 ♂	2 ♂	2 ♂	1 ♂	1	1	1	0	0	0	0	0	0d					6	3	9	14

some extent degenerate in structure. The females are somewhat flask-shaped, flattened dorso-ventrally, and become pigmented as they age. The males, on the other hand, are nearly cylindrical, transparent, and are somewhat shorter and about one-half the width of the newly-hatched female. The toes of the female are long and slim, those of the male are short and thick. The lorica of the male, especially in the posterior region, shows a greater degree of segmentation than that of the female, and the body is thus more flexible. One of the most striking peculiarities of the male rotifer is a black, disk-shaped body. It consists of a vesicle containing large, dark granules, which are supposed by Leydig to be excretory in nature (Wesenberg-Lund, 1929, p. 327). This structure is located dorsally in the posterior region of the body.

The females more than double their size through growth (compare Figs. 2 and 3); the males do not grow. This lack of the power of growth is characteristic of the males of most species of rotifers and is associated with the absence of a functional digestive system. In *Lecane inermis*, no traces of the mastax and digestive glands, so prominent in the female, have been observed in the male. Apparently, only the rudimentary anterior portion of the digestive tube persists, and this serves, as in other species (Wesenberg-Lund, 1923, 1929), as a suspensor ligament for the testis (compare Figs. 3 and 5).

The excretory system shows some structural reduction in the absence of the contractile vacuole.

The reproductive system is well-developed. The testis is large and fills most of the body cavity. The vas deferens is also conspicuous. Two large bodies lying ventral to the testis and vas deferens may be the so-called prostate glands, two or four of which are usually present in the male rotifers. The structure and method of functioning of the copulatory organ has not been observed.

A prominent retro-cerebral organ and eye-spot are present, as in the female. The activity and flexibility of the male indicate well-developed nervous system and musculature.

The males hatch from the small eggs of the mictic female after a developmental period somewhat exceeding in duration that of the females. Four male parthenogenetic eggs, allowed to develop in standard oat infusion at 23° C., required two hours longer to develop than four female parthenogenetic eggs laid at the same time and subjected to identical conditions during development. The average time required by the male eggs was 31 hours 16 minutes, by the female eggs 29 hours 45 minutes, a difference of one hour and 31 minutes. In a suspension of *Chlorella* and *B. proteus* in Benecke solution of

0.07 per cent concentration, at 22°–24° C., the male embryos require on the average four hours longer for hatching than the female embryos. The duration of the embryonic periods of male and female parthenogenetic eggs deposited by young parents and allowed to develop at the same time are given in Table XI.

TABLE XI

Lecane inermis. Comparison of the duration of the embryonic periods of contemporaneous male and female parthenogenetic eggs, deposited by young mothers, and allowed to develop at 22° to 24° C. The eggs were isolated in pairs on hollow-ground slides, usually a single male and female egg in each depression, in a suspension of *Chlorella vulgaris* and *B. proteus* in Benecke solution of 0.07 per cent concentration.

Number of hours after deposition	Number of contemporaneous eggs hatched	
	Female	Male
30	18	0
32	6	0
34	1	7
36	1	10
38	0	9
39—or longer	0	6
	Total 26	32

Male rotifers have been reported to be sexually mature at the time of hatching or shortly thereafter. This is true also of the males of *Lecane inermis.* During the first few days they are extremely active. They copulate indiscriminately with fertilized or unfertilized mictic females, or with the amictic females. This seems to be generally characteristic of rotifer males. The duration of sexual activity has not been investigated.

The males that hatch live usually 4 to 6 days. They are very active. They swim dorsal side up, commonly in a less direct path, and rotate less than do the females. The males of this species are also typical in the lack of the power of growth. On the fourth or fifth day, activity begins to wane until locomotion ceases entirely. The organism lies on its side in a semi-contracted condition, the anterior portion bent ventrally. This condition culminates in death usually within 24 hours.

For investigation of the length of life and rate of mortality, male eggs were isolated within 24 hours after deposition, from small mass cultures during a period of three weeks. These individuals, as well as the mictic and amictic populations previously described, belonged to the same pure line (descendants by parthenogenesis of a single stem mother). Of the 110 eggs isolated, 6 individuals that hatched were lost in transferring, and 22 (20 per cent) failed to hatch. Nine of those that failed to hatch were discarded before it was discovered that in many cases the embryos developed completely and were very active within the shell. Only one of the remaining 13 failed to develop. The other 12 individuals lived and were active the usual length of time within the shell; two lived longer than any of those that hatched.

Table XII

Lecane inermis. Length of life of 95 males, including 13 which developed but failed to hatch. The length of life is reckoned from the time of isolation of the eggs (within 24 hours after deposition). For comparison of the males with the females, the differences in the biometric constants of the different populations are given at the end of the table. The biometric constants for the female populations are given in Table 11.

Number of days	1	2	3	4	5	6	7	8	Total
Those that hatched	0	1	1	4	20	50	6	0	82
Those that developed but failed to hatch	1	0	0	0	1	7	2	2	13

	Mean	Standard Deviation	Coefficient of Variability
Total studied (95)	5.69 ± 0.07	1.10 ± 0.05	17.48 ± 0.88
Total hatched (82)	5.65 ± 0.06	0.83 ± 0.04	14.73 ± 0.79

Difference between Males and Females			
Amictics (108)—Males (95)	3.21 ± 0.13	0.72 ± 0.09	1.80 ± 1.27
Mictics (111)—Males (95)	5.41 ± 0.29	3.37 ± 0.21	21.93 ± 2.22

Table XII shows the life duration of those males, hatched and not hatched (95 individuals), for which records are complete. They lived from one to 8 days. Sixty per cent of the population died on the sixth day, which was the modal length of life. Twenty-two per cent died on the fifth day and only a very few on any other day. The mean length of life of those that hatched is 5.65 days; including those that failed to hatch the mean is 5.69 days. The males live three days less than the amictic females, five and one-half days less than the mictic females. The standard deviation is significantly lower than that of both types of females; the coefficient of variability is only slightly less than that of the amictic females, but less than that of the mictic females by 22 per cent.

In Fig. 10, the rate of mortality of male and female populations is compared. Those that developed but failed to hatch are included in the graph. The rate of mortality increases very gradually until the fourth day, at the end of which seven and one-half per cent of the males, but no females are dead. During the fifth and the sixth days, 88 per cent of the male population die, while mortality is just beginning in the female population. All of the males are dead one day before mortality reaches its height in the female population.

The male and female populations differ in hatchability, in the mode and mean length of life, and in the standard deviation; but in the variability of life duration the males and the amictic females do not differ appreciably.

Is the difference in hatchability of the male-producing and female-producing eggs due to an inherent difference in fertility of the mictic and amictic females, or to differences in the ages of the parents or to the conditions of cultivation? The males were, for the most part (77 per cent), derived from females under four days old; all the females studied were deposited by individuals less than four days old. Most of the male-producing eggs that failed to hatch were derived from parents more than four days old, but six were early-born. Thus, 7 per cent of the early-born males and only 0.5 per cent (one in 220) of the early-born females failed to hatch. The female embryos that fail to hatch never live more than one or two days. Since the female and male populations were not cultivated at the same time, however, the failure of male embryos to hatch may have resulted from some environmental difference, and cannot be regarded as conclusive evidence that mictic females produce parthenogenetic eggs that are regularly less fertile than those from amictic females. Information regarding the relative fertility of the parthenogenetic eggs from mictic and amictic females is not available in the literature, in so far as I am aware.

The uniformly short length of life of the males in comparison with that of the females has been observed in other lines investigated on a smaller scale, in which, however, both sexes were cultivated at the same time.

The males of *Lecane inermis* live longer than the males of most other species hitherto described (Wesenberg-Lund, 1930, p. 158). The large males of the viviparous *Asplanchna* species live four or five days. The moderate reduction in length of life, in comparison with that of the females, is, in *Lecane inermis*, in accord with the moderate degree of structural degeneracy. Differences in the degree of structural degeneracy of the males of different species may bear some correlation with differences in the degree of reduction in the size of the male-producing parthenogenetic eggs and in the rate at which the eggs mature and are deposited, two factors which Wesenberg-Lund (1930) considers to be largely responsible for the structural degeneracy of the male rotifers. Evidence in support of this view is derived from a comparison of the males of *Lecane inermis* with those of the plankton rotifers. The former, which are characterized by a moderate degree of structural degeneracy and of reduction in length of life, are derived from eggs which are appreciably smaller than the female-producing eggs, but which are deposited less rapidly, and require a longer time for development. The males of the plankton rotifers, on the other hand, are derived from very small eggs, a large number of which, according to Wesenberg-Lund, are produced almost simultaneously, and develop rapidly. These males are small, consist of little more than reproductive system and live (in some cases) only a few hours. It should be borne in mind, however, that the tendency toward structural degeneracy and reduced vigor of the male rotifers may result from the haploid condition of their chromosomes (Morgan, 1926, Chapter X). The knowledge regarding these matters is at present very meager.

SUMMARY

This paper is the first contribution of a series dealing with the life cycle of the bisexual rotifer *Lecane inermis* Bryce. The three types of individuals, the mictic females, the amictic females and the males, and the eggs from which they are derived, are described and their life histories are compared statistically. The individuals studied are intrinsically uniform members of the same genetic stock, cultivated under uniform environmental conditions, although they were not all contemporaneous. The comparison is based upon about 100 representatives of each type of individual.

The three types of individuals are characteristically different in length of life. The mean length of life is, for the amictic females, 8.9 ± 0.11 days, for the mictic females, 11.1 ± 0.28 days, for the males, 5.7 ± 0.07 days. This difference is apparent in their life curves. Embryonic mortality is negligible in all three populations. Mortality increases gradually in the male population until the fourth day, then increases suddenly, 83 per cent of the population dying on the fourth to the sixth days. All are dead by the eighth day. All the females survive through the fourth day; thereafter the rate of mortality increases more rapidly in the amictic population. More than half of the mictic females survive the modal life duration, which is the same for both kinds; 12 per cent to 15 per cent survive the longest-lived amictic female and die off very gradually until the twenty-eighth day. The short life duration of the males is undoubtedly correlated with the structural degeneracy, especially of their digestive and excretory systems.

The longer life of the mictic females, as compared with the amictic females, results, in part at least, from the fact that egg-production is for them a less strenuous process. This is evidenced by the following facts. (1) Their male-producing eggs are smaller. (2) The mictic female produces regularly only two-thirds as many eggs as the amictic female. The maximum number is, for the mictic female, 16, exceptionally 17, for the amictic female 24. The mean number of eggs per individual is, for the mictic female 14.2 ± 0.11, for the amictic female, 20.7 ± 0.13. A higher proportion of mictic females produce their maximum number of eggs. The difference between the mode and the maximum is, for the mictics, only one, for the amictics, three. (3) The mean duration of the period of fecundity of the amictic female is 6.4 ± 0.09 days, with a standard deviation of 1.44 ± 0.07 and a coefficient of variability of 22.5 per cent; the mean for the mictic female is 5.1 ± 0.05 days, the standard deviation 0.83 ± 0.04, the coefficient of variability 16.2 ± 0.75 per cent. (4) During this time the mictic female deposits, on the average, one egg every 8.6 hours, the amictic female deposits one egg every 7.5 hours. (5) The amictic females die usually within 24 to 36 hours after deposition of the last egg; 19 per cent of the mictics lived more than six days thereafter. In summary, the mictic female produces in less rapid succession only two-thirds as many eggs as the amictic female, requires for the production of her eggs 1.3 days less than the amictic female and usually lives longer after the cessation of egg-production.

A comparison was made, for the mictic and amictic females, of the degree of correlation between length of life and their fecundity, rate of egg-production and ability to survive the fecund period. Short-lived amictic females are found to have produced fewer eggs than long-lived individuals, in more rapid succession, and to have died within a few hours after the deposition of the last egg, apparently exhausted by the severity of the process of egg-production. The length of life of the mictic female is correlated only in slight degree with the number of eggs produced and the rate at which they are produced.

The mictic female of this species may be fertilized during immaturity or after having produced parthenogenetic eggs. The fecundity of the mictic female is reduced by fertilization, the total number of eggs per individual, in 18 cases studied, not exceeding ten. The maximum number of fertilized eggs produced by a single individual was five. The length of life of the mictic female is not appreciably altered by the production of fertilized eggs.

The differences cited above are evidence of fundamental physiological diversity between mictic and amictic females and males of *Lecane inermis.*

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PERMEABILITY AND FATIGUE IN MUSCLE AND ITS BEARING ON THE PROBLEM OF ION ANTAGONISM

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It has been shown by numerous investigators that stimulation of different tissues leads to an increase in permeability which is independent of the particular structure and function.[2] In the case of muscle, Weiss showed that even subminimal stimuli lead to an increase in permeability which was measured by an increase in the output of phosphoric acid by the muscle. When effective stimuli were applied, a close relationship between the intensity of fatigue and the increase in permeability was observed (Embden and Adler, 1922). Furthermore, there was a parallelism between the reversibility of fatigue and that of the changes in permeability. When the muscle recovered from fatigue and the original contractility was again obtained, the permeability was also normal, *i.e.*, no phosphoric acid was given off to the medium in which the muscle was suspended. But if no recovery took place, the output of phosphoric acid remained the same or even increased. These experiments prove that the physico-chemical changes in permeability play an important part in the fatigue problem. It seemed probable that fatigue might be delayed if it were possible to prevent or to diminish the increase in permeability which ordinarily accompanies fatigue. The experiments described in this paper show the correctness of the supposition. Fatigue can be delayed in a medium by which the permeability of the muscle is decreased. The efficiency of different ions in that respect and the general importance of these facts for the problem of ion antagonism will be discussed in this paper.

METHOD

Several hundred experiments on the sartorius muscle of *Rana esculenta* were performed from March 1929 to October 1930. The muscles, prepared in the usual manner, were immersed in an isotonic salt solution and suspended between two platinum electrodes connected with an isotonic lever which magnified the contractions seven times.

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[1] Awarded the A. Cressy Morrison Prize in Experimental Biology for 1930 by the New York Academy of Sciences.

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[2] Compare Gellhorn, 1929, pp. 166–197.

In the first group of experiments the platinum electrodes were carefully covered with lacquer (except on a point-like spot where the platinum was in contact with the muscle). The muscles were stimulated with rhythmical condenser discharges (apparatus of Scheminsky) 40–60 times per minute so that a decrease in the height of contraction occurred. The two sartorii of the same frog under the same conditions showed an identical course of fatigue (Gellhorn, 1930). This made it possible to study the influence of different ions. The solutions were made up of NaCl, KCl and $CaCl_2$ solutions of $\triangle = 0.39^\circ$. In order to hold the conductivity of the liquid constant and avoid any possible effect on the strength of the stimulus, a part of the sodium chloride solution was replaced by an equal volume of isotonic $CaCl_2$ solution, and thus the effect of different $CaCl_2$ concentrations was studied.

In a second group of experiments another method was used. The sartorii were not stimulated in a liquid but in a moist chamber. The first series of stimuli was applied for five minutes and the muscle was then allowed to recover in an aërated salt solution. Afterward a second series of stimuli was applied until the muscle ceased to respond. The first series of stimuli was designed to show whether both sartorii had the same irritability and fatigability. Pairs of muscles which behaved alike in the first series of stimulation were the only ones considered as material for the determination of the influence of different salt solutions. The effect of these solutions was apparent in the course of fatigue during the second series of contractions. The advantage of this second method consists in the independence of the strength of the stimulus from the conductivity of the solution and furthermore in the exact proof of the identical behavior of the muscles before they were immersed in the salt solutions. In these experiments condenser discharges obtained from the apparatus of Scheminsky (1930) in its improved form were used. Maximal stimuli were given 90 times per minute. Between the first and the second series of stimulations the muscle remained unstimulated for nine minutes in the salt solutions. The experiments of the second group were performed with curarized and uncurarized muscles. The effect of Ca, Sr, Ba, and Mg was studied in reference to the fatigue of muscle.[3]

Experiments Performed with the First Method

Figure 1 shows the course of the height of contractions of the sartorii stimulated 40 times per minute for twelve minutes. One muscle was in NaCl + KCl + $CaCl_2$ solution in which these salts had

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[3] After preparation the muscles remained in aërated Ringer's solution for 45–60 minutes before the experiment was begun.

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the same concentration as in Ringer's solution, while the other muscle was bathed in $NaCl + KCl + CaCl_2$ which contained eight times as much $CaCl_2$ as the first one. It is apparent that, in spite of the identical height of the contractions at the beginning of the experiment, both

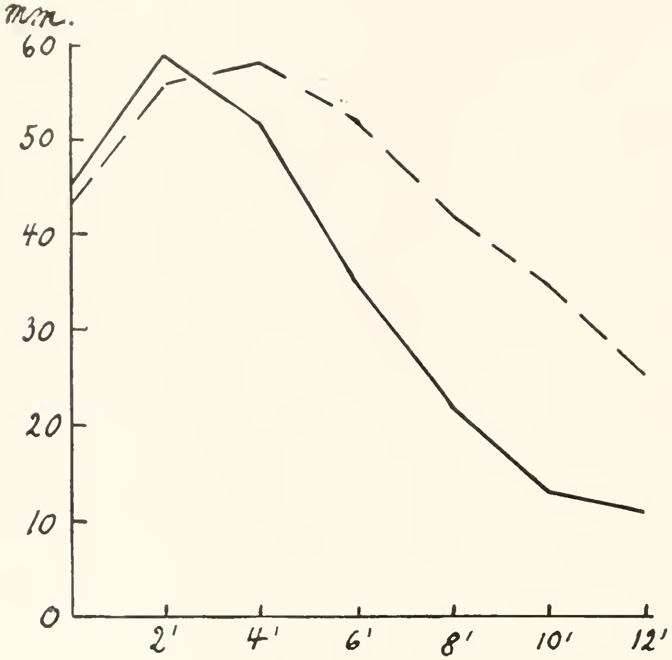

Fig. 1. The course of fatigue in a pair of sartorii stimulated in 0.111 M $NaCl + 10.2\ M \times 10^{-4}\ KCl + 8.1\ M \times 10^{-4}\ CaCl_2$ (——) and in 0.103 M $NaCl + 10.2\ M \times 10^{-4}\ KCl + 64.4\ M \times 10^{-4}\ CaCl_2$ (– – –).

Ordinate: height of contraction; Abscissa: time in minutes.

Maximal condenser discharges; frequency 40 per minute.

show the "treppe" in the same amount:—the muscle immersed in a solution with a high $CaCl_2$ concentration showed a lesser fatigue than the control muscle. In other words *an excess of $CaCl_2$ delays fatigue.* This fact, which was the basis for further experimentation, is illustrated by Fig. 2, which reproduces a series of experiments performed with six pairs of sartorii. The control muscle was always stimulated in 0.111M $NaCl + 0.00102M\ KCl + 0.00081M\ CaCl_2$, the other muscle in 0.00486M or 0.00972M $CaCl_2$, the KCl concentrations being identical in both cases and the NaCl slightly less in order to keep constant the osmotic pressure and conductivity of the solution. The contractions were registered at intervals of two minutes for a period of twelve minutes and the height of contraction of the muscle stimulated in the

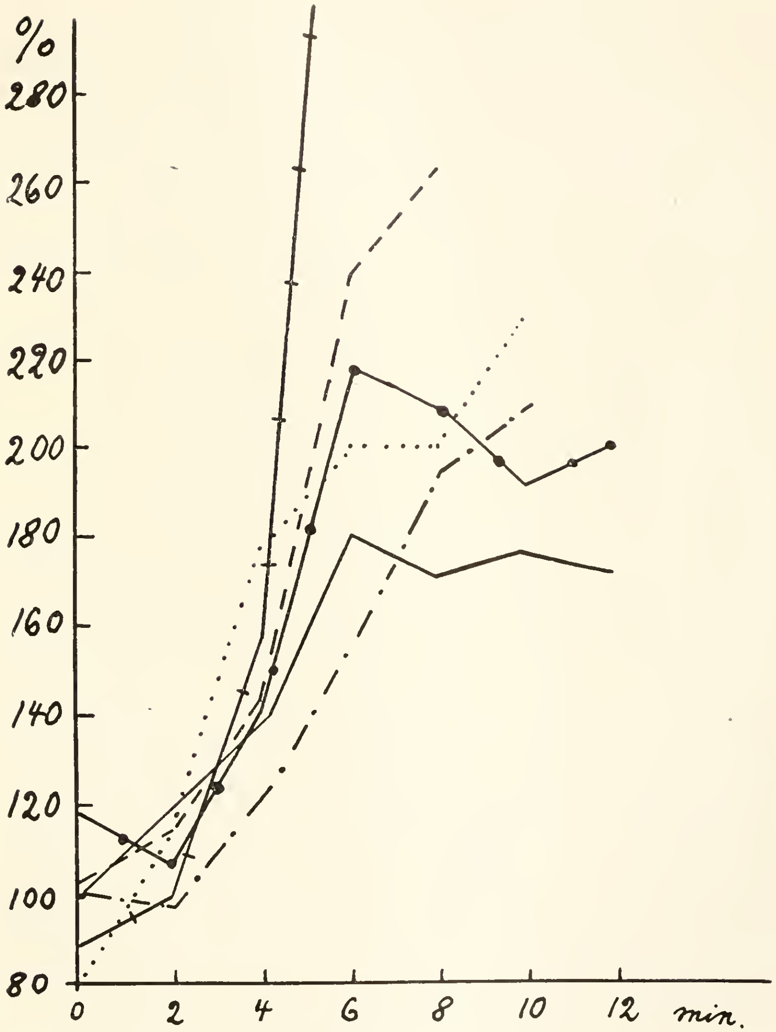

FIG. 2. The influence of different $CaCl_2$ concentrations on the course of fatigue in sartorius muscle. The height of contraction of the control muscle (stimulated in 0.111 M NaCl + 10.2 M × 10^{-4} KCl + 8.1 M × 10^{-4} $CaCl_2$) is taken as 100 and the percentage of the height of contraction of the muscle placed in a solution with higher $CaCl_2$ concentration is calculated.

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———●——— } 0.106 M NaCl + 10.2 M × 10^{-4} KCl + 48.6 M × 10^{-4} $CaCl_2$.
— — — — —

— · — · —
. . . . } 0.0996 M NaCl + 10.2 M × 10^{-4} KCl + 97.2 M × 10^{-4} $CaCl_2$.
—|——|——

higher $CaCl_2$ concentration was calculated as the percentage of the height of contraction of the control muscle. The figure shows that the height of contraction in the beginning of the experiment exhibited very slight variations if any, but in the further course of the experiment the deviations became progressively stronger and reached in most cases 100 per cent or more. This illustrates again the delay of fatigue in solutions with higher $CaCl_2$ concentrations and shows at the same time that the increase of $CaCl_2$ to twelve times its normal concentration (*i.e.*, the concentration in Ringer's solution) is still favorable for the preservation of contractility and the delay of fatigue.

The fundamental importance of this fact is apparent when the effect of the same solutions is studied on the irritability of the unstimulated muscle. As was to be expected, the solution with the "normal" $CaCl_2$ concentration is much more favorable to the preservation of irritability than a solution with higher $CaCl_2$ content (Fig. 3). Therefore the optimal conditions are different for the stimulated and unstimulated muscle. That is to say, *the laws of ion antagonism which lead to the discovery of the equilibrated solutions do not hold quantitatively for the stimulated muscle.* These experiments make probable an even

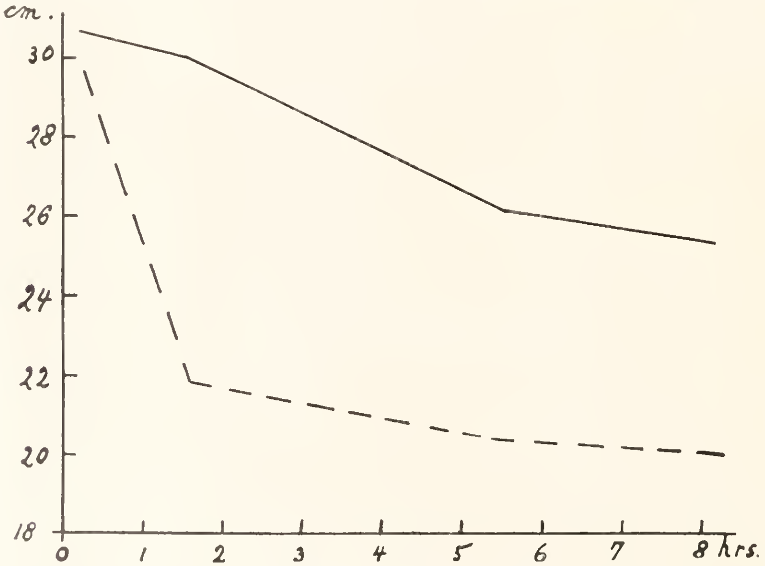

FIG. 3. The course of irritability (threshold) in unstimulated muscles. Ordinate: Distance between primary and secondary coil; abscissa: Time in hours.
———0.111 M NaCl + 10.2 M × 10^{-4} KCl + 8.1 M × 10^{-4} $CaCl_2$.
— — —0.103 M NaCl + 10.2 M × 10^{-4} KCl + 64.4 M × 10^{-4} $CaCl_2$.

more general formulation that the efficiency of ions on cells depends upon their degree of permeability.

Experiments Performed with the Second Method

In order to check these results more than 100 experiments were performed with the second method. Figure 4 illustrates the course of fatigue after the muscles have been immersed in solutions of different $CaCl_2$ concentrations. The curves I and II show the fatigue curve of the two muscles when stimulated in the moist chamber. One recognizes that they behave identically. After this they were immersed for nine minutes in solutions of $NaCl + KCL + CaCl_2$ which were alike save for the $CaCl_2$ concentration. This was $8.1\ M \times 10^{-4}$ in the first case and $81\ M \times 10^{-4}$ in the latter one. Finally they were trans-

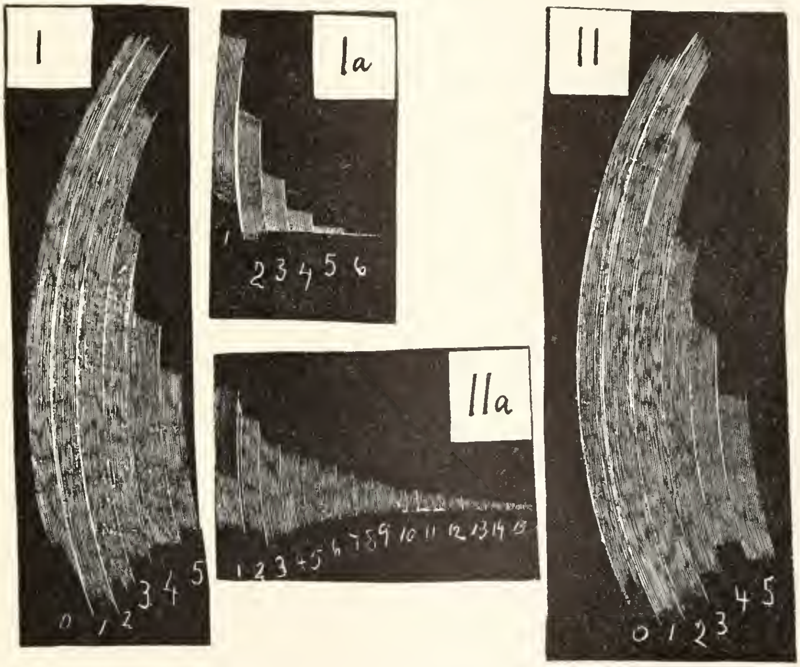

FIG. 4. Stimulation of a pair of sartorii in a moist chamber with maximal condenser discharges 90 times per minute. Then muscle I is immersed in $0.111\ M\ NaCl + 10.2\ M \times 10^{-4}\ KCl + 8.1\ M \times 10^{-4}\ CaCl_2$ (control fluid) and muscle II in $0.111\ M\ NaCl + 10.2\ M \times 10^{-4}\ KCl + 81\ M \times 10^{-4}\ CaCl_2$ for nine minutes. Thereafter stimulation in a moist chamber as before (curve I*a*, II*a* respectively). The numbers at the curves indicate the time in minutes after the beginning of stimulation.

ferred again into the moist chamber and stimulated in the same way as in the first series. The experiments yielded the curves I*a* and II*a* and prove that the solution rich in $CaCl_2$ preserved the contractility of the muscle much better than that with the lower $CaCl_2$ concentration. The height of contraction which was observed in the first muscle (treated with 8.1 M × 10^{-4} $CaCl_2$) after five minutes was the same as in the second muscle (treated with 81 M × 10^{-4} $CaCl_2$) after fourteen minutes.

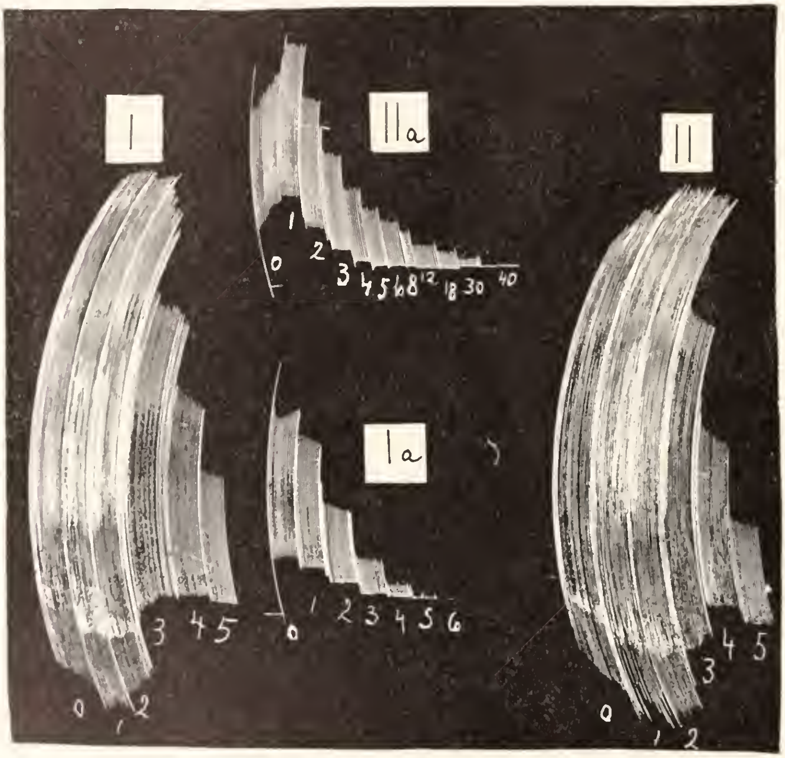

FIG. 5. Procedure as in Fig. 4. Muscle I immersed in control fluid; muscle II in 0.111 M NaCl + 10.2 M × 10^{-4} KCl + 40.5 M × 10^{-4} $CaCl_2$.

Another example is reproduced in Fig. 5. It is of particular interest because in the first series of contractions the second muscle shows spontaneously a greater fatigability than the first one. Nevertheless, the treatment of the muscle with the solution containing a higher $CaCl_2$ concentration overcompensated the deficiency, since this muscle remains contractile for 40 minutes and the control muscle for only five minutes. In a group of experiments the solution with the higher $CaCl_2$ concentration contained the same NaCl concentration as the other one so that the osmotic pressure of the first solution was slightly higher, while in a second group the NaCl concentration was a little lower in order to keep constant the osmotic pressure in spite of the differences in $CaCl_2$ concentration. In both groups the solution rich in $CaCl_2$

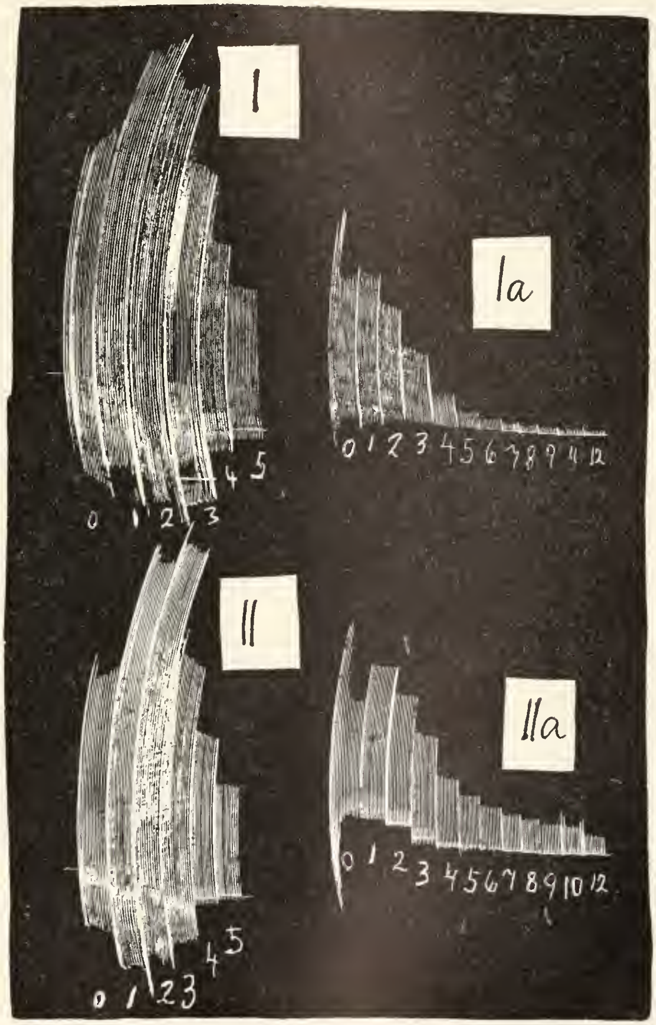

FIG. 6. Procedure as in Fig. 4. Muscle I (Fig. 1 and 1*a*) immersed in control fluid; muscle II in 0.111 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 40.5 M $\times 10^{-4}$ $CaCl_2$ (Fig. II and II*a*).

delayed the fatigue, and this result was also obtained if by addition of $NaHCO_3$ the salt solution were completed to a typical Ringer's solution. Figure 6 shows this in a very striking way because, in spite of the weaker contractions of the second muscle, the fatigue is delayed after the muscle has been immersed in a Ringer's solution with five times its normal $CaCl_2$ concentration.

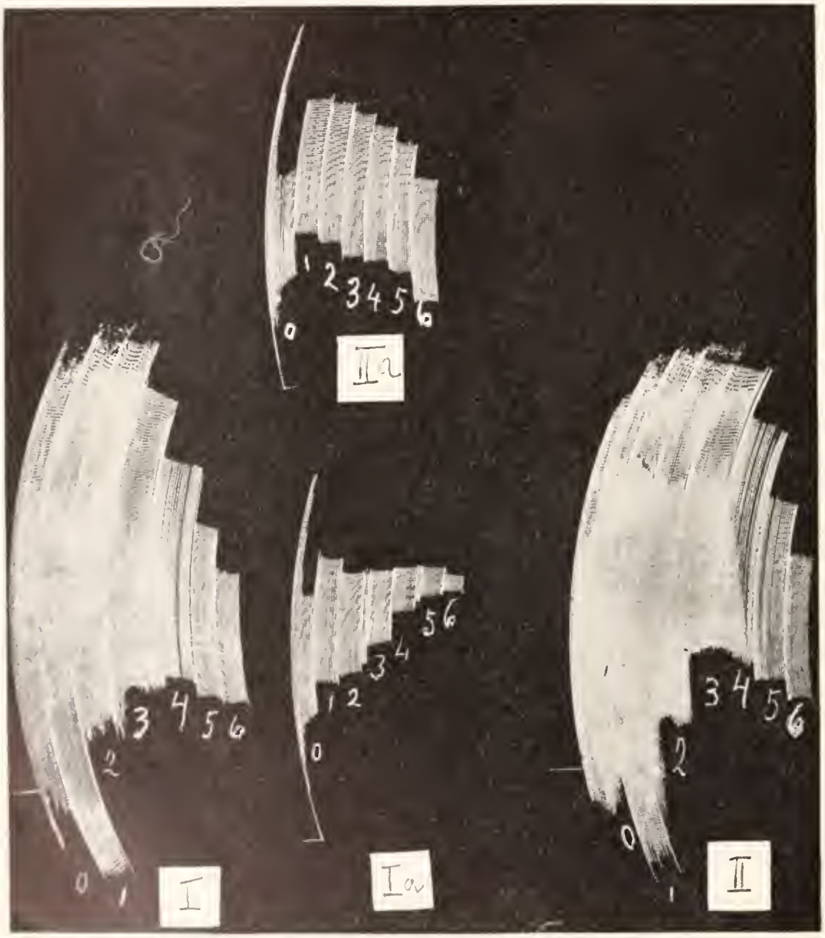

Fig. 7. Procedure as in Fig. 4. Muscle I in 0.111 M NaCl + 10.2 M × 10^{-4} KCl + 81 M × 10^{-4} $CaCl_2$ (I and I*a*); muscle II in control fluid (II and II*a*).

The favorable effect of the solution with higher $CaCl_2$ concentration in delaying fatigue may be illustrated by Table I, in which the time of the preservation of contractility was determined for each pair of muscles after they had been immersed in salt solutions with different $CaCl_2$ concentrations.

TABLE I

The Dependence of the Duration of Contractility upon the $CaCl_2$ Concentration

After a previous period of stimulation for five minutes, the muscles were immersed in 0.111M NaCl + 0.00102M KCl + different $CaCl_2$ concentrations as indicated in this table. After this, stimulation was continued in a moist chamber.

No.	$CaCl_2$ Concentration	Duration	$CaCl_2$ Concentration	Duration
		minutes		minutes
1	$8.1M \times 10^{-4}$	5	$40.5M \times 10^{-4}$	> 17
2	"	5	$40.5M \times 10^{-4}$	> 40
3	"	6	$81M \times 10^{-4}$	> 15
4	"	4	$81M \times 10^{-4}$	> 9
5	"	4	$81M \times 10^{-4}$	46
6	"	6	$81M \times 10^{-4}$	20

The experiments with the second method reveal still another fact which seems to be of interest for the problem of the tonus of skeletal muscle. The curves of Fig. 5 in particular show that after the inter-

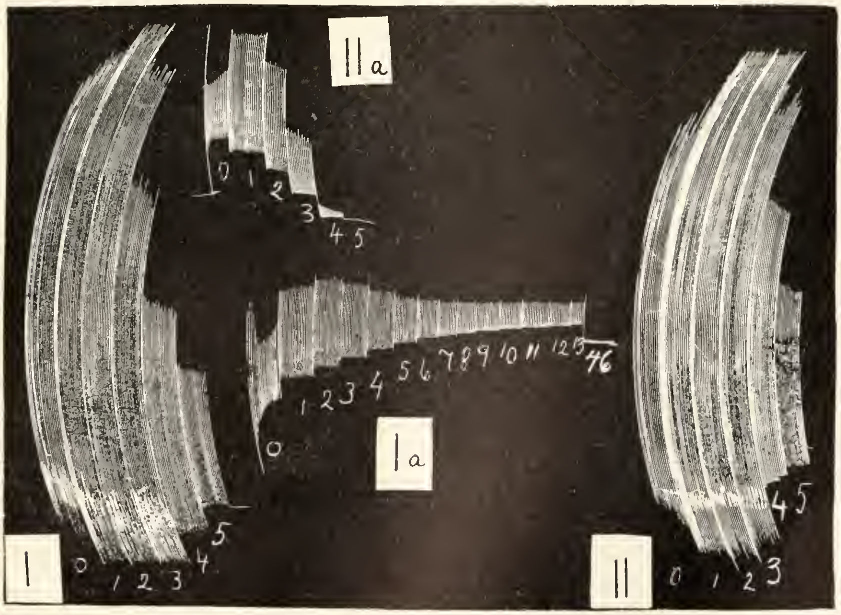

FIG. 8. Procedure as in Fig. 4. Solutions as in Fig. 7.

val during which the muscles have been immersed in salt solutions the stimulation leads to a contracture which is slightly stronger in the Ca-muscle. Furthermore, the diminution of this contracture is delayed in the Ca-muscle.[4] In this experiment the contracture still increased one minute after the beginning of stimulation while in the control muscle it diminished. This behavior is even more pronounced in the experiment illustrated by Fig. 8, in which the Ca-muscle showed an increase in tonus during almost the entire period of stimulation, although the control muscle showed a rapid loss of tonus during this time. The tonus effect of calcium becomes apparent when the $CaCl_2$ concentration is 81 M $\times$ 10^{-4} or higher. In reference to the delay of fatigue the muscles show an unequal behavior. In most cases the fatigue was delayed in spite of the increase in tonus, but in some cases (compare Fig. 7) the tonus increased so rapidly that it led to an acceleration of fatigue.

The behavior of the stimulated and unstimulated muscle in solutions containing different $CaCl_2$ concentrations was compared and it was concluded that stimulation entirely changed the reactivity of the muscle to this particular ion. This fact was explained by the increase in permeability of the muscle. The observations on the effects of calcium ions on the tonus of skeletal muscle seem to support this view since Neuschlosz (1922) observed that the immersion of the *resting* muscle in Ringer's solution with increased $CaCl_2$ concentration lead always to a *decrease* in tonus. It is probable that the muscle brought into a state of high permeability by previous stimulation takes up $CaCl_2$ in a greater amount than the resting muscle and this leads to entirely different phenomena. It may be emphasized that about the same concentrations were used in Neuschlosz' experiments and our own. If the interpretation is correct it is to be expected that far greater concentrations of $CaCl_2$ would be able to increase the tonus in the resting muscle. In fact it was found by Guenther (1905) that $CaCl_2$ in one per cent solution leads to an increase in tonus which occurs after a latent period of several hours. Probably the irritability of the muscle had faded under these conditions. In contrast to that, the experiments described above show that $CaCl_2$ may immediately raise the tonus of the irritable muscle provided that the permeability had been increased by previous stimulation. Therefore not only by K as Neuschlosz found, but also by Ca the tonus of muscle can be increased without decrease of irritability.

Leaving aside the effects of $CaCl_2$ on the tonus of muscle, the experiments lead to the conclusion that increase in $CaCl_2$ concentration through a very wide range reduces the higher degree of permeability produced by stimulation, and therefore delays fatigue. Further experiments dealt with the question whether the Ca effect is specific or whether it is replaceable by other cations. The effects of Sr, Mg and Ba were examined.

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[4] For the sake of brevity the expression Ca-muscle is used for the muscle which has been immersed in the solution containing the higher $CaCl_2$ concentration.

The experiments showed that *none of the alkali earths can replace calcium.* Rather small concentrations of Sr, Ba and Mg had only a slight effect if any, and greater concentrations accelerated fatigue. $MgCl_2$ seemed to have the most harmful effect, since it reduced the life duration of the muscle which had been previously immersed in a solution of $NaCl + KCl + MgCl_2 + NaHCO_3$, while the effect of Sr and Ba was mostly limited to a decrease in the height of concentration. The concentrations used are given in Table II.

TABLE II

Each solution ordinarily contains beside the salts with bivalent cations 0.111M $NaCl + 10.2M \times 10^{-4}$ $KCl + 12.5M \times 10^{-4}$ $NaHCO_3$.

	Concentration	Effect on Fatigue
Mg	$15.4M \times 10^{-4}$	—
	$23.1M \times 10^{-4}$	—
	$30.8M \times 10^{-4}$	—
Ba	$23.9M \times 10^{-4}$	—
	$31.8M \times 10^{-4}$	—
Sr	$16.7M \times 10^{-4}$	—
	$32.5M \times 10^{-4}$	—
	$40.7M \times 10^{-4}$	—
	$81.4M \times 10^{-4}$	—

In another series one muscle was immersed in a solution of $NaCl + KCl + CaCl_2 + NaHCO_3$, the composition of which was identical with that used for the control muscle save for the addition of $SrCl_2$, $BaCl_2$ or $MgCl_2$. In these experiments it was found that all three cations were able to delay the fatigue of the muscle but the effect was much less than in the experiments described above in which an excess of $CaCl_2$ was studied. Furthermore, it was found that the favorable effect was limited to a very small range of concentrations. When this was surpassed the fatigue was accelerated. The quantitative data are reproduced in Table III.

TABLE III

The Effect of Sr, Ba and Mg on the course of fatigue if added to $0.111M\ NaCl + 10.2M \times 10^{-4}\ KCl + 8.1M \times 10^{-4} CaCl_2 + 12.5M \times 10^{-4}\ NaHCO_3$.

	Concentration	Effect on Fatigue *
Sr	$32.5M \times 10^{-4}$	+
	$40.7M \times 10^{-4}$	+
Ba	$19.8M \times 10^{-4}$	+
	$23.9M \times 10^{-4}$	+
	$31.8M \times 10^{-4}$	−
Mg	$15.4M \times 10^{-4}$	+
	$19.2M \times 10^{-4}$	±
	$30.8M \times 10^{-4}$	−

* + indicates delay in fatigue; −, acceleration in fatigue; ±, no change.

DISCUSSION

The experiments described in this paper prove that the laws of ion-antagonism differ quantitatively in the resting and the stimulated muscle. The increase in permeability in the latter leads rapidly to fatigue and this can be delayed when an excess of Ca is added which reduces permeability. On the other hand, a Ringer's solution containing an excess of $CaCl_2$ is unfavorable for the preservation of irritability in *resting* muscle. The fundamental difference in the behavior of resting and stimulated muscle in reference to ions is further illustrated by experiments in which the effect of other bivalent cations was studied. It was found that Sr, Mg or Ba added to Ringer's solution with normal $CaCl_2$ concentration exerted a favorable influence on the stimulated muscle, that is, the fatigue was delayed. But the same solutions were unable to preserve the irritability of the unstimulated muscle as well as Ringer's solution. The experiments permit one to conclude that *the composition of the optimal salt solution is not a definite one for a certain type of cell but is dependent upon its degree of permeability.*

This implies the fact that the efficiency of ions differs quantitatively if applied to a cell in a state of low or high permeability. The experiments on the tonus effect of calcium described above show the correctness of this conclusion. The increase in tonus observed in the stimulated muscle begins immediately after stimulation, while, according to Guenther, it requires a Ca concentration ten times higher and several hours in resting muscle.

The experiments throw an interesting light upon the specificity of ion antagonism.[b] They indicate that, in contrast to the ion antagon-

[b] Compare Gellhorn, 1924 and 1926.

ism between K and bivalent cations in muscle in which numerous cations are of great antagonistic efficiency, calcium cannot be replaced by any other bivalent cation in its effect of delaying fatigue. This is astonishing because the suppression of the K contracture by bivalent cations is based upon their power to reduce permeability (Gellhorn, 1928). The writer found recently in experiments on vital staining in sea urchin eggs that the permeability of the cell to stains could be prevented by Ca but not by Mg, Sr or Ba (1931). But the explanation of the specificity seems to be quite different in sea urchin eggs and muscle, since in the former Sr, Ba and Mg were unable to decrease permeability, while in muscle permeability is decreased. This is not only indicated by observations on K-contracture but also by the fatigue experiments of this paper, since it was shown that addition of Sr, Ba or Mg to Ringer's solution containing $CaCl_2$ was effective in reducing fatigue. The reason why Ca cannot be entirely replaced by Sr, Ba and Mg seems to lie in the fact that Ca is probably the only ion which diminishes the increase in permeability of muscle in a completely reversible manner. However, when coarser changes in the surface of the cell are concerned, such as are produced by K, several bivalent cations act antagonistically. Fewer of these ions are effective if irritability is concerned, as in paralysis of muscle (Höber, 1917), than in the case of K-contracture, which is rather independent of irritability. In the former case Ca is the most antagonistic ion, in the latter case the heavy metals (Gellhorn), because here the greater the power in reducing permeability, the more efficiently is the contracture suppressed. The impossibility of fine reversible changes in permeability, which are necessary for the preservation of irritability, is not concerned here. The intermediate case, the restoration of irritability after K-paralysis, is influenced in a somewhat intermediate manner: calcium is most effective, but other bivalent cations are effective to a lesser degree. The importance of calcium for the preservation of irritability and contractility is apparent. It is conceivable that the delay of fatigue requires a still finer adjustment than the partial restoration of irritability after K-paralysis. This may be the reason for the specificity of the calcium effect in the fatigue experiments of this paper.

SUMMARY

1. The fatigue of the sartorius muscle of the frog can be delayed by an excess of calcium chloride in Ringer's solution, although this solution does not preserve irritability of resting muscle as well as Ringer's solution. Therefore, in contrast to the view generally accepted today, *the quantitative composition of the optimal solution depends upon the permeability of the cell.*

2. This conclusion implies a quantitative difference in the efficiency of ions in relation to permeability. This is illustrated by observations on the increase of muscle tonus in solutions with high $CaCl_2$ content. The change in tonus takes place in irritable muscle.

3. The effect of calcium on fatigue is specific. Neither Sr, Ba nor Mg can replace it. But these cations can delay the fatigue, although to a lesser extent than calcium, when added to Ringer's solution containing $8.1 \text{ M} \times 10^{-4}$ $CaCl_2$. The cause of this specificity is discussed.

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THE RECOVERY CONTRACTURE IN MUSCLE; A NEW GENERAL SALT EFFECT[1]

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In a study on permeability and fatigue in muscle and its bearing on the problem of ion antagonism, the method used in a series of experiments was as follows. The sartorius muscle was stimulated for five minutes with condenser discharges about 60–90 times per minute. Thereafter the muscle was allowed to rest for at least five minutes in Ringer's solution with normal or increased Ca content and then a new series of stimulations of the same frequency was begun. The muscle was in a moist chamber when stimulated. Under these conditions a peculiar phenomenon was observed. In the beginning of the second stimulation series the muscle showed a contracture which became less during the continuation of the stimulations and finally disappeared more or less completely. The phenomenon is fundamentally different from the fatigue contracture in muscle which increases with the continuation of stimulations. The experiments described below serve to analyse the phenomenon.

METHOD

The experiments were carried out on m. sartorius and biceps of *Rana esculenta*. The carefully prepared muscles were suspended between platinum electrodes and stimulated by means of the apparatus of Scheminsky, which allowed one to use condenser discharges over a wide range of frequencies. The strength of the stimulus was varied by a parallel resistance; there was also a resistance of 10,000 ohms in series in order to make very slight changes in the conductivity of the muscle ineffective. The stimuli were either just maximal or submaximal. The muscles were loaded with 2 g; the magnification of the isotonic lever was six fold. The experiments were performed from October 1930 to January 1931.

RESULTS

I. THE BASIC PHENOMENON

A typical example is reproduced in Fig. 1. In this experiment the sartorius was stimulated 90 times per minute with a condenser of 0.5 mf. Between the periods of stimulation the muscle recovered in a

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[1] Aided by a grant from the research fund of the University of Oregon.

well-aerated Ringer's solution. Each period of stimulation and recovery lasted for five minutes. One recognizes from Fig. 1 that the curves *b*–*d* show in the beginning contractures which decrease while stimulation is being continued. There is a regular decrease in the height of contracture from *b* to *d*.[2] This makes it plain that the phenomenon in question is basically different from the fatigue contracture since (1) fatigue contracture increases with the continuation of stimulation and (2) in spite of a slight decrease in the height of contractions from *b* to *d*, *i.e.*, an increase in fatigue, the contracture becomes less. This peculiar behavior of the contracture makes it rather probable that it might be due to a recovery and not to a fatigue process. As to the height of contractions, it is characteristic that although the contractions decrease in the very beginning of the contracture until the maximum of the contracture has been reached, hereafter a remarkable increase in the height of contractions is accompanied by a decrease in contracture.

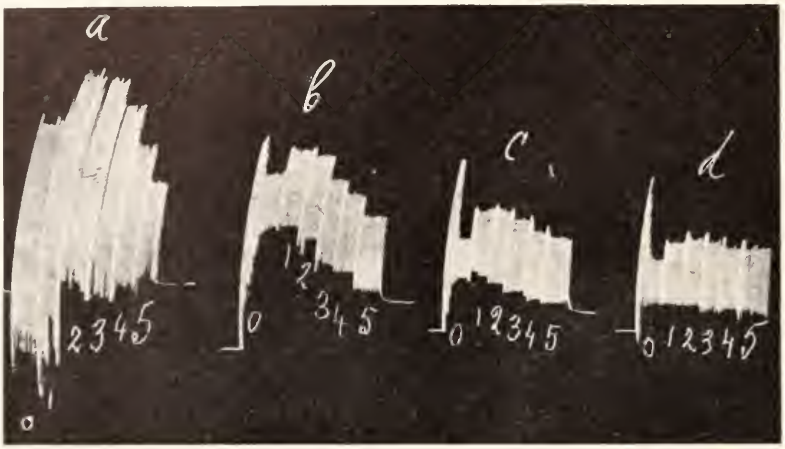

FIG. 1. Four stimulation periods on sartorius. Condenser discharges (0.5 mf.) 90 times per minute, in a moist chamber. Muscle is in Ringer's solution during recovery period. The conditions in all succeeding experiments are the same unless otherwise specified

There are still other facts which support the hypothesis that the phenomenon is a recovery contracture. It is known that with increasing fatigue the duration of the contraction increases. The detailed study has shown that the change in duration chiefly concerns relaxation. At first one may see that an elastic vibration ends the period of relaxation. With increasing fatigue the elastic vibration disappears and a slow contraction (designated as "Funke's Nose") is apparent in the downstroke of the contraction. The more fatigue progresses the earlier in the downstroke appears this slight contraction. Since the relaxation period of the "Funke's Nose" is much longer than in a normal contraction the duration of the contraction increases in correspondence with the earlier appearance of the "nose." The registration of the recovery contracture on a faster revolving drum revealed the interesting fact that the changes described as characteristic for fatigue (Funke, Wachholder) also occur during the recovery contracture but in the *reverse order* as is shown in Fig. 2, in which tracings obtained at intervals of one minute are recorded.

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[2] In the text and in the figures the different stimulation periods are designated as *a*, *b*, *c*, *d*. The first which can show recovery contracture is the *b* curve.

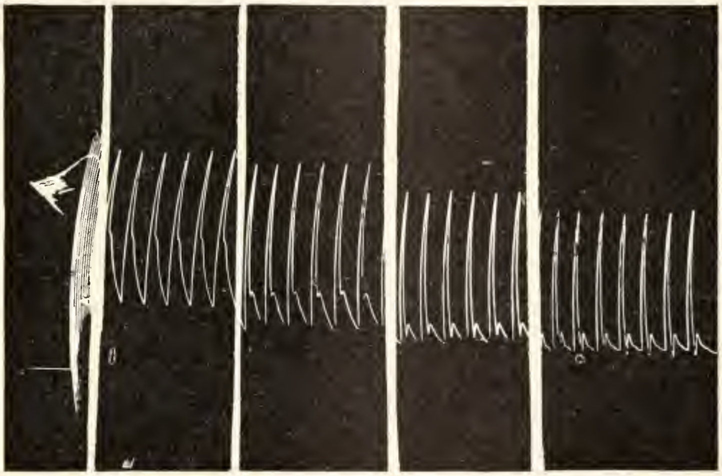

FIG. 2. *b*-curve on a faster moving drum.

It was stated above that the recovery contracture decreases with increasing number of alternating stimulation and recovery periods as is apparent if the recovery contracture in Fig. 1, *b* is compared with 1, *c* or 1, *d*. Experiments on a fast revolving drum revealed that the changes in shape and duration of the contraction correspond to simultaneous changes in the size of contracture. In an experiment, for instance, which was carried out in a similar manner as that reproduced in Fig. 1, it was found that the disappearence of the "Funke's Nose" required one minute in experiment *d*, four minutes in experiment *c* and more than five minutes in experiment *b*. In other words, the recovery phenomenon judged in its intensity according to the height of contracture and to the time during which the "Funke's Nose" appears decreases the more the fatigue increases. That is, of course, to be expected, since recovery is more marked in a slightly fatigued muscle than in one which has been very much fatigued. Thus the phenomenon is undoubtedly a recovery contracture and the question arises as to what factors are responsible for its occurrence.

II. The Influence of Strength, Frequency, and Duration of Stimuli on the Occurrence of Recovery Contracture

Systematic studies showed that the recovery contracture is rather independent of the strength of the stimuli since it occurs in experiments with submaximal as well as maximal condenser discharges. This makes it plain that the contracture is different from Tiegel's contracture, which requires the application of supermaximal currents. However, the frequency of stimulation had a marked influence. Experiments were carried on in which the frequency of stimuli in the first and second periods varied independently over a range of from 30 to 150 per minute. It was apparent that at least 45 stimulations were required in the first period to bring about the contracture in the second, provided that during this period the frequency was at least one hundred. Under these conditions, the higher the frequency employed in the second period, the more marked the contracture. But this is true only up to a frequency of 120 per minute, since beyond this value the contracture dropped slightly. It is easily understandable that stimulations below 45 per minute during the first period did not produce a recovery contracture in the second even if very high frequencies were used, since under these conditions in the first period no fatigue was observed. Therefore, a recovery phenomenon must fail to appear.

In general it may be said that the higher the frequencies used in both periods, the more marked the recovery. But even after a first period of high frequency the contracture does not occur unless the mus-

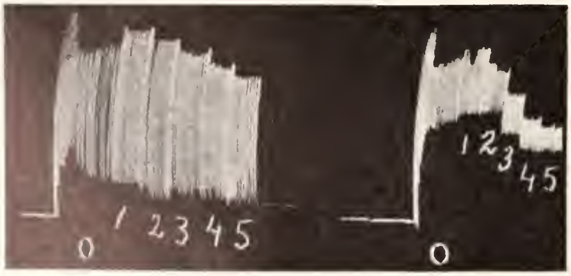

Fig. 3. *b*-curve in two sartorii of the same frog. Frequency of stimulation in the first *b*-curve 60 per minute; in the second, 120 per minute.

cle is stimulated at least 45 times per minute in the second period. The strength of the recovery contracture is not only apparent in its height but also in the changes in the shape and duration of the contraction. This may be illustrated by Figs. 3 and 4. In Fig. 3 the recovery contracture of two sartorii of the same frog are reproduced. The frequency during the first period was the same in both cases (90 per minute), while in the second period the first muscle was stimulated 60 times and the second muscle 120 times per minute. Fig. 4 shows that the "Funke's Nose" disappears in the downstroke of the contraction within one minute, frequency of stimulation being 60 in the first and 90 in the second period, while if during both periods the muscle is stimulated 90 times per minute, it takes much longer for the duration of contraction to become normal. (Compare Fig. 2.)

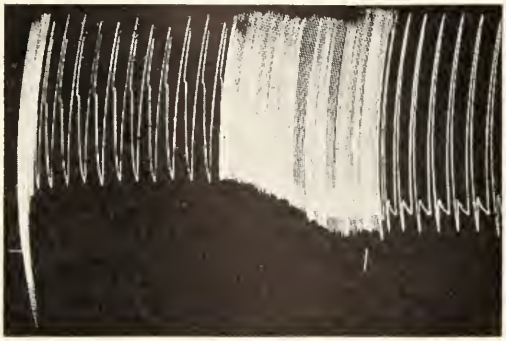

FIG. 4. *b*-curve. Frequency in *a*-curve was 60; in *b*, 90 per minute.

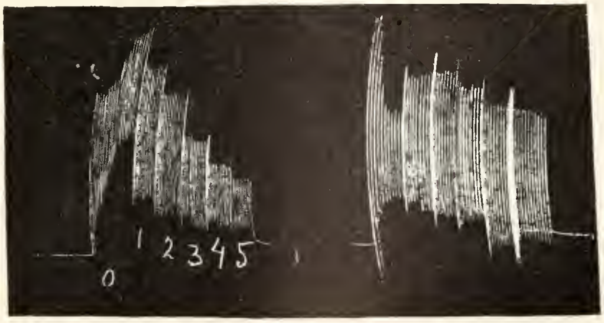

FIG. 5. *b*-curve. The two sartorii were stimulated in the *a* period with a condenser 0.1 mf.; in the *b* period of the first muscle a condenser of 2.0 mf. was used, in that of the second a condenser of 0.1 mf.

The duration of the stimulus also seems to affect the recovery contracture. In Fig. 5 the recovery contracture of two sartorii of the same frog is reproduced from an experiment in which strength and frequency of the stimulus were identical, but the duration of the stimulus was in the first muscle 70σ and in the second 20σ. One recognizes in Fig. 5 that increasing duration of the stimulus increases recovery contracture. But even with very short currents the phenomenon can be observed, since recovery contracture is obtained in sartorius after application of submaximal induction currents. It also shows under these conditions the characteristic features mentioned above which concern the occurrence of the "Funke's Nose" and the duration of the single contraction.

III. The Influence of Salts and Non-electrolytes on the Occurrence of Recovery Contracture

This paragraph deals with the problem of the physico-chemical factors which determine the occurrence of the recovery contracture. In the first group of experiments one muscle recovered in the moist chamber while the other muscle of the same frog recovered in Ringer's solution as in the previously described experiments. In order to get

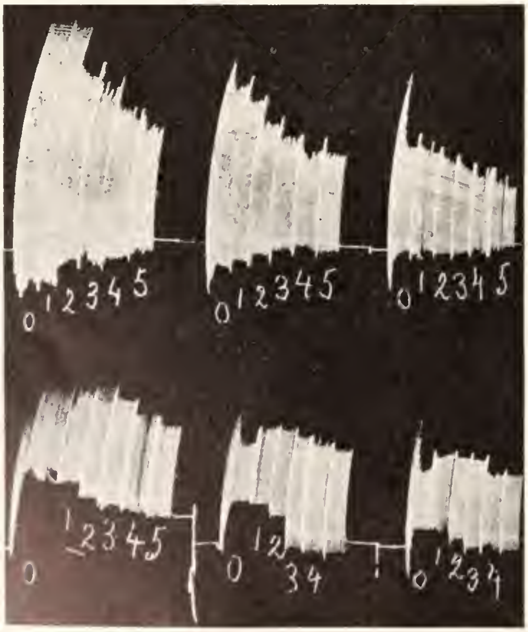

Fig. 6. *b-d* curves of two sartorii of the same frog. Upper tracing: recovery period in moist chamber. Lower tracing: recovery period in Ringer's solution.

distinct results it is advisable to avoid soaking the muscle in Ringer's solution for a long time when it is afterwards to be in the moist chamber during the recovery period.

Dulière and Horton observed recently that a muscle which is brought into a moist chamber without having been in contact with Ringer's solution lost its irritability. The observations of these authors were confirmed and therefore the muscle was immersed in Ringer's solution for two minutes before being placed in the moist chamber. Under these conditions the irritability of the muscle did not change and the very striking result was regularly obtained that the muscle which recovered in a moist chamber did not show recovery contracture, while the muscle immersed in Ringer's solution during recovery period always showed the contracture. Figure 6 gives an illustration of the typical results for three periods (*b-d*). While the "Ringer" muscle undergoes the typical recovery contracture, decreasing more and more with each stimulation period, the "air" muscle does not show any contracture.[3] The change in the shape and duration of the contractions is also restricted to the muscle which recovered in Ringer's solution and showed recovery contracture.

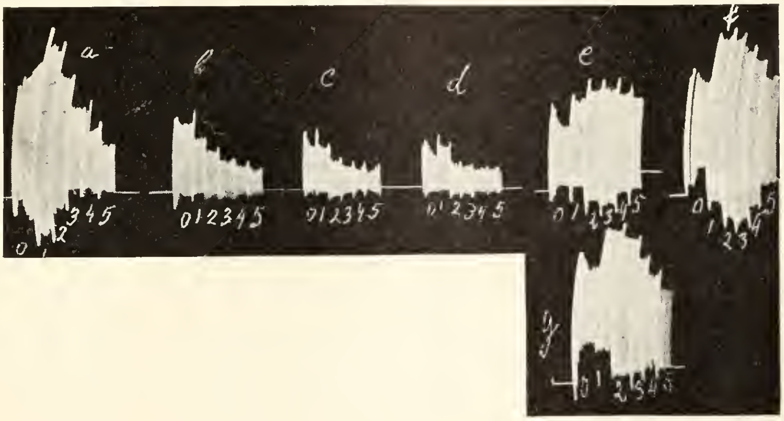

FIG. 7. *a–g* curves of sartorius. Between *a* and *d* period, muscle in moist chamber; between *d* and *g*, in Ringer's solution during recovery.

Experiments of this type were performed in great number without a failure. It is interesting to note that, as Fig. 7 indicates, alternating conditions bring about alternate presence and absence of the contracture of the muscle. In this experiment the muscle remained during the first three recovery periods in a moist chamber and therefore did not show a recovery contracture; after the fourth stimulation period it always remained in Ringer's solution during the recovery period. The effect is very significant. The long period in air influenced the behavior of the muscle so that one recovery period in Ringer's did not immediately bring about the contracture. Therefore it is not noticeable in experiment *e*, but it becomes apparent in *f* and still more in *g*.

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[3] For the sake of brevity the muscle which remained in Ringer's during the recovery period is referred to as the "Ringer muscle," that which remained in the moist chamber, the "air muscle."

The time during which the muscle was kept in Ringer's solution before the experiment was started is not immaterial to its reaction in reference to the recovery contracture. Thus it was found that soaking the muscle for 24 hours in Ringer's solution increases its tendency to react with recovery contracture even if the muscle is kept in a moist chamber during the recovery period. This may be illustrated by Fig. 8. The first curve showed a typical although very slight recovery contracture. But the contracture was still smaller in the second sartorius of the same frog, which was held in a moist chamber for one hour before starting the stimulation. It was, of course, also soaked for 24 hours in Ringer's solution. Besides, the second muscle shows but a slight decrease in the elastic vibrations during contracture while the first one did not show them at all. This indicates, as was proven by registering on a fast revolving kymograph, that in the first case the contracture was accompanied by the occurrence of the "Funke's nose," which did not occur in the second case. Summing up these observations, it may be said that recovery contracture occurs if the muscle is kept in Ringer's solution during the recovery period and it disappears if the muscle is in a moist chamber during that period. The effects are reversible. The soaking of the muscle in Ringer's solution furthers the recovery contracture.

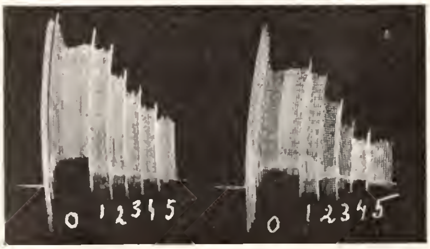

FIG. 8. Both sartorii soaked for 24 hours in Ringer's solution. First muscle immediately thereafter stimulated as in Fig. 1, the second muscle remained one hour in moist chamber before stimulation. Muscles in moist chamber during recovery period. *b*-curves reproduced.

Seeking for an explanation of this peculiar phenomenon it seemed rather probable that the presence or absence of the contracture might be due to differences in ion concentrations at the surface layer of muscle, since the efficiency of ions to produce contractures and influence the "tonus" of muscles is known (Neuschlosz, Gellhorn). But experiments did not prove the correctness of this view. The recovery contracture occurs not only if the muscle is kept in Ringer's solution but also if the composition of that solution is much altered. An increase in the K or Ca concentration to ten times its original value did not noticeably influence the strength of the recovery contracture. The phenomenon was also observed in isotonic NaCl, Na_2SO_4 and LiCl solutions. If the K or Ca content of the muscle were responsible for the phenomenon in question, changes in concentration of these ions should be effective. Therefore it was thought that one might have to do here with a general salt effect as was first observed by Loeb (for further references compare Gellhorn, 1926 and 1929, pp. 153–163). In this case the contracture should be diminished or should not occur at all if the muscle were placed in a non-electrolyte solution during the recovery period. In fact, the experiments carried out with glucose, sucrose and urea either alone, in isotonic solution or replacing different parts of Ringer's solution, yielded the expected results. Figures 9 and 10 show a typical experiment on sartorius and biceps. In Fig. 9 the recovery contracture was much decreased if the muscle remained in a solution consisting of Ringer's + urea during the recovery period. In Fig. 10 it is shown that recovery contracture is suppressed completely if the muscle is immersed in isotonic sucrose solution during the recovery period.[4]

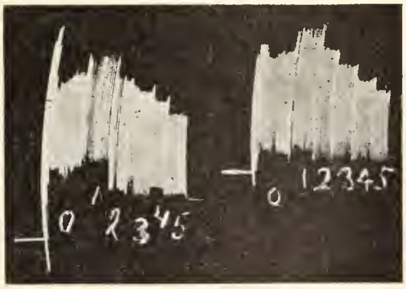

FIG. 9. *b*-curves. (m. sartorius.) Muscle 1 in Ringer's, muscle 2 in 3 cc. Ringer's + 17 cc. isotonic urea during recovery period.

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[4] In some of the experiments it was observed that the sartorious muscle showed a small contracture when immersed in isotonic non-electrolyte solutions. Therefore in most cases mixtures of Ringer's and non-electrolyte solutions were employed which did not produce a contracture during the recovery period.

As mentioned above, the experiments were carried out on the sartorius and biceps of *Rana esculenta.* The results were the same in both muscles. This seems of interest in respect to observations of Sommerkamp, who found that some groups of muscles react more to reagents which produce contractures, especially to acetylcholin, than others. This fact was explained by structural differences because the content of "tonusfibers" is different in different muscles. The observations of this paper seem to indicate that in the recovery contracture only the ordinary muscle fibers are involved since biceps and sartorius behaved rather alike in spite of the differences observed by Sommerkamp in experiments with acetylcholin.

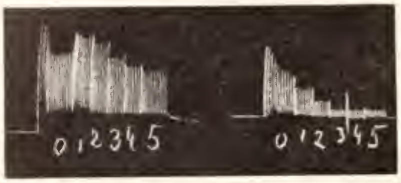

FIG. 10. *b*-curves. (m. biceps.) Muscle 1 in Ringer's, muscle 2 in isotonic sucrose during recovery period.

As to the explanation of the recovery contracture, several facts support the assumption that the condition of the surface layer of the muscle is the determining factor for its appearance. This is not meant in the sense of the theory of Botazzi, who believes that sarcoplasm is responsible for "tonic" reactions, but there are a number of observations which show more or less the dependence of contractions and contractures upon the surface layer of the muscle cell and its permeability. It will be recalled that fatigue in muscle can be delayed by an excess of calcium in Ringer's solution (Gellhorn), and furthermore, that K and SCN contractures depend entirely on permeability (Gellhorn).

The importance of the behavior of the surface of muscle for the recovery contracture is expressed by two groups of observations: 1. It was shown that the recovery contracture is increased by the application of condenser discharges with relatively long duration of discharge. As was recently proved by v. Gulacsy and Heller such discharges bring about marked polarisation phenomena on the surface layer of muscle. 2. Experiments were described demonstrating that the recovery contracture was diminished or suppressed in the presence of non-electrolytes. This shows that a general salt effect is present as in the experiments of Loeb. It still remains a question as to whether the same process underlies the salt effect in Loeb's and in our own observations. But the experiments seem to point out that the conditions of the colloids in the surface layer of the cells are different when recovery contracture occurs and when it is prevented.

SUMMARY

If the sartorius or biceps of *Rana esculenta* is stimulated with condenser discharges in periods of five minutes with a frequency varying between 45 and 150 per minute and stimulation periods alternate regularly with recovery periods of the same duration, a recovery contracture is brought about in the beginning of the second and each following stimulation periods provided the muscle is allowed to recover in an aerated salt solution. Wide changes in the K and Ca concentration of the Ringer's solution are without influence. The contracture also occurs when isotonic solutions of Na or Li salts are employed. The phenomenon is suppressed by replacing the salt with a non-electrolyte. The following observations are in favor of the assumption that the contracture is a recovery contracture:

1. The phenomenon does not occur if the frequency is too low to produce even a slight fatigue.
2. It decreases from the second to the fourth stimulation period, *i.e.*, with increasing fatigue.
3. The changes in the shape of the contractions (Funke's Nose) during the recovery contracture show a temporal sequence just the opposite of that in fatigue.

It is assumed that changes in the surface layer of the muscle cell bring about the recovery contracture. In favor of this hypothesis are the facts:

1. The recovery contracture is increased with increasing duration of the electrical discharges which were used to stimulate the muscle.
2. Non-electrolytes which suppress the phenomenon bring about changes in the surface layer of cells.

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NOTE ON DR. JAN HIRSCHLER'S PAPER ON SPINDLE BRIDGES, AND THE *DE NOVO* ORIGIN OF MOTH SPERMATOCYTE GOLGI BODIES

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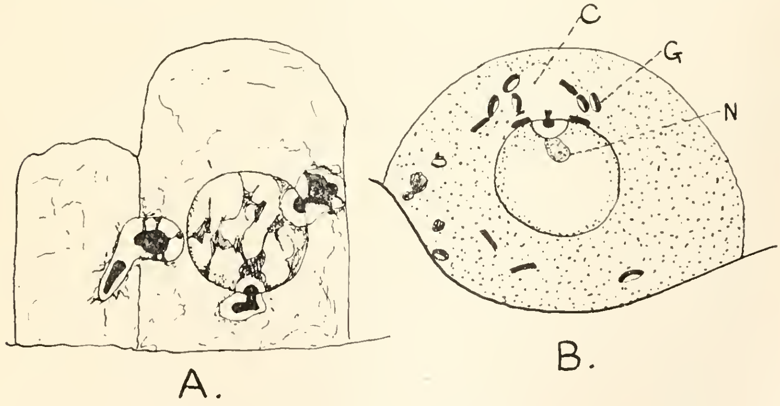
Introduction

In a recent paper Jan Hirschler (1929) has described fully in *Macrothylacia rubi* L., a remarkable relationship between certain cytoplasmic elements, and the nucleus. He states that he has found this in other genera of Lepidoptera, namely, *Phalera*, *Dasychira*, and *Dendrolimus*.

Hirschler's technique consisted in Zenker fixation followed by iron alum staining, and chrome-osmium acid fuchsin for Golgi bodies. Apparently the Zenker method shows no cytoplasmic inclusions but does show very remarkably a connection between hemispherical nucleoli and spindle bridges, such as does not appear to have been described before. In text figures A and B are reproductions of two



TEXT FIGS. A AND B
After Jan Hirschler (1929*a*)

A. Zenker fixed cells showing spindle bridges and attachments to nucleoli ("tres analogues aux hétérochromosomes d'autres Insectes").

B. Osmic Golgi preparation, showing Golgi bodies (*G*) originating around a spindle bridge space (*C*). Ordinary nucleolus, *N*. Both figures from *Macrothylacia rubi*.

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[1] Theresa Seessel Fellow, Osborn Zoölogical Laboratory, Yale University.

of Hirschler's figures illustrating in the first, Zenker hæmatoxylin prepared cells with two curious hemispherical nucleoli at the nuclear membrane, each connected with an extension into the cytoplasm, described by Hirschler as "résidus fusoriaux." The nucleoli he claims to be analogous with the heterochromosomes of other insects. Prepared by an osmic Golgi method, one gets the picture shown in text figure B. The hemispherical nucleolus with its pale halo, has a small object protruding from it into the cytoplasm; a normal or ordinary nucleolus (*N*) adheres to the hemispherical one. Now, just in this region there is a pale zone (*C*) surrounded by Golgi bodies. Hirschler says, "Nous considerons cette zone comme le résidu fusorial et nous pensons que, sous l'influence de la substance nucléaire qu'elle renferme et qui touche au cytoplasme, il se forme dans celui-ci, par précipitation des éléments de l'appareil de Golgi qui peuvent ensuite perdre contact avec le résidu fusorial et se transporter en d'autres régions de la cellule."

In a previous paper (Gatenby, 1931*a*) the present writer has referred to the same idea of Hirschler, by which a new formation of secondary Golgi bodies is supposed to take place even in the spermatids of a pentatomid. This hypothesis of Hirschler (1929*b*) has been dealt with in the previous paper, and is considered untenable so far as it applied to insect germ cells studied by the present writer.

The Material and Methods.—The material used in the present work consisted of a large series of normal, x-radiated and radium-radiated testes of *Abraxas*. No material from other moths is at present available, but a large number of lepidopterous testes from other families has been studied in recent years.

The Spindle Bridge in Abraxas.—In Fig. 2 of the plate is a group

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DESCRIPTION OF PLATE

Letters: B, bacteria. *BR*, branch of spindle bridge. *D*, dead cell. *GB*, Golgi body. *L*, living cell. *SB*, spindle bridge. *X*, dying cell. *Y*, normal-looking cells.

All *Abraxas* and, except Fig. 3, by Champy hæmatoxylin.

FIG. 1. Group of spermatocytes, some normal-looking (*Y*), one dying (*X*), as the result of x-radiation.

FIG. 2. Rosette of spermatogonia showing live and dead cells as result of gamma-radiation. Spindle bridge at *SB*. Such cells already contain Golgi bodies as in next figure.

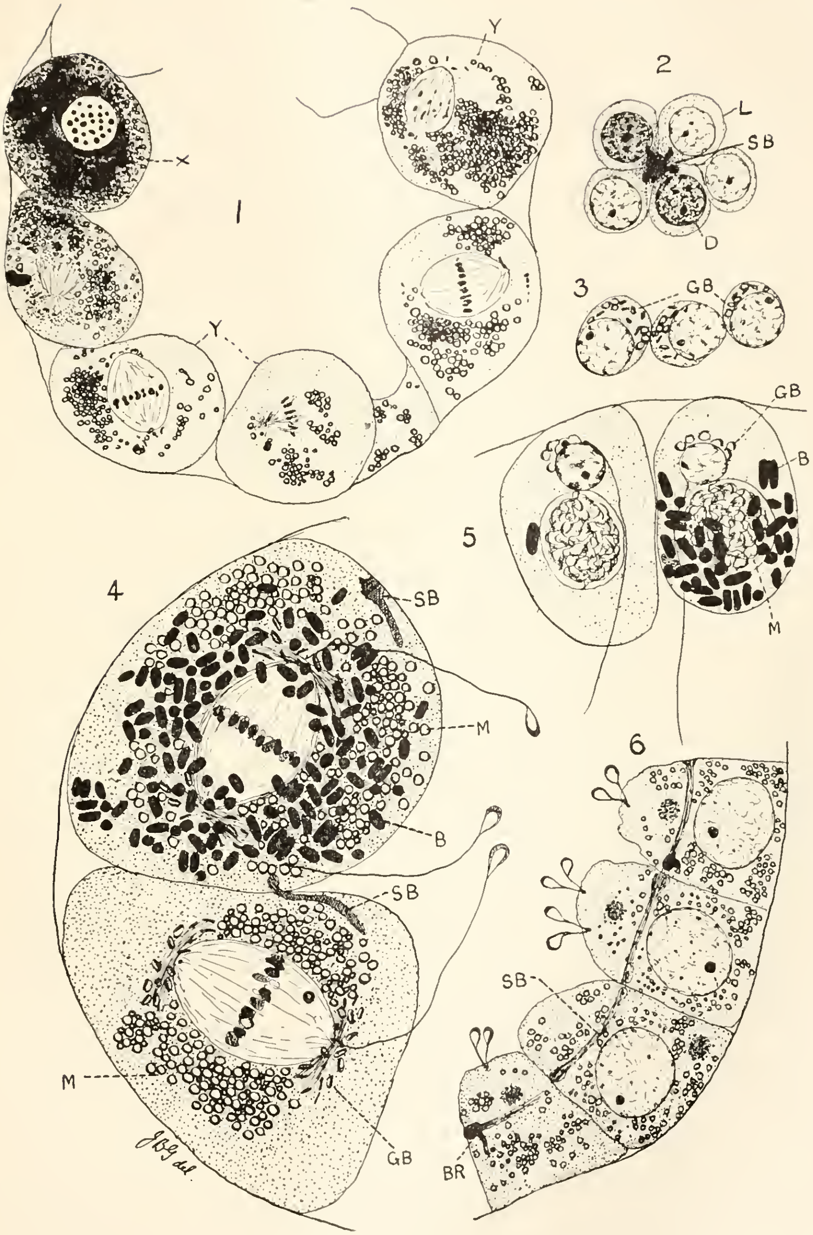
FIG. 3. Nassanow method. Rosette spermatogonia with numerous Golgi bodies. These cells are much smaller than those in text figures A and B, in which Golgi bodies are supposed to be arising *de novo*.

FIG. 4. Two spermatocytes, with metaphase spindles, showing dense bacterial infection in one, and freedom from infection of the other.

FIG. 5. Two spermatids, one badly infected, the other containing one bacterium.

FIG. 6. Group of growing spermatocytes showing spindle bridges, mitochondria and neutral red vacuoles (just above the bridges).

PLATE I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of spermatogonia connected by blackly-staining spindle bridges. When the cells hollow out to form the typical nest of growing spermatocytes, each unit is found to be connected to its neighbour by long lighter-staining bridges which pass right across the cytoplasm, in front of the nuclei, as shown in Fig. 6, *SB*. In no case has the spindle bridge been found to lie in any other region.

In spermatogonia and young spermatocytes, the spindle bridges stain sharply in chrome-osmium material which has been fixed overnight and well washed out. As the cells become larger the spindle bridges become more difficult to find under the Champy or F. W. A. hæmatoxylin treatment. It is only in special regions that the bridges are shown as clearly as in Fig. 4, at *SB*, in full grown spermatocytes.

Now in no case, in chrome-osmium hæmatoxylin preparations, could the structure depicted in text figure A be found. The spindle bridges were "solid" and never appeared to consist of two parts, inner chromophile region, and an outer canal-like structure. It was also in vain that in the chrome-osmium hæmatoxylin slides the modified heterochromosomes (hemispherical nucleolus) were looked for. In the case of the Kolatschew preparations nothing corresponding to the hemispherical nucleolus was found. The writer is certain that the ordinary nucleoli of *Abraxas* are not heterochromosomes (Gatenby, 1931*b*), and has found as in Fig. 6, that they lie in all directions in the nuclei, some towards, others away from the spindle bridge (*SB*). Very careful examination failed to reveal any side branch coming from the spindle bridge towards the ordinary nucleolus. Of course, it is quite possible that Champy's fluid and iron alum hæmatoxylin may have failed to show both the side branch and the hemispherical nucleolus. It is not thought that Jan Hirschler's figures are not correct, but it can only be stated that *Abraxas* must be very different from the forms studied by him.

Then to refer to text figure B—nothing like this has ever been found in *Abraxas* except in the beginning stages of the formation of abortive acroblasts (Gatenby, Mukerji, and Wigoder, 1929), which was a purely experimental phenomenon.

The Origin of Golgi Bodies in Moths.—Hirschler makes the remarkable statement that he believes that Golgi bodies appear in *Macrothylacia*, under the influence of nuclear substance, on the edge of the spindle-bridge shown in text figure B at *C*. Here again the present writer finds this somewhat difficult to credit. In Fig. 3 of the plate are three very small spermatogonia from rosettes; these cells already contain Golgi bodies. All the evidence which the writer could find seems to indicate that Golgi bodies are to be seen in the smallest rosette cells in young testes, and do not originate as claimed by Hirschler. Nor do the ordinary nucleoli in such cells bear any relationship to the spindle bridges, the confines of which (Fig. 2) are clearly demonstrable.

The Function of the Spindle Bridges.—It seems certain that the function of the spindle bridges in such animals as the moths, is to keep all the cells in one nest at approximately the same stage of growth and physiological condition. For in the testis crowded with all stages of spermatogenesis, some cells lying nearer the testicular wall are better exposed to nutriment than others.

That there must be some sort of connection between the cells of a nest seems well shown by the "experiment" in Fig. 4. *Abraxas* is often infected with large bacteria which live in many of the cells of the body, and quite often in the testis. Here are two spermatocytes in first maturation division—both at the same stage, but one cell is simply crammed with bacteria, so as almost to hide the spindle. Now it appears unlikely that a cell so burdened could keep up with its neighbours unless it received active help from them. Yet such infected cells go successfully through maturation and produce spermatids as in Fig. 5, which are perfectly normal except for the bacteria. Finally, the latter come to lie on the sperm tails and are probably instrumental in spreading the infection to non-infected eggs. In testis cells, such bacteria are not symbiotic.

Radiation and Cell Nests.—Radiation by either x- or gamma-rays, provides another aspect to this question, and seems to show that the injury caused by this type of treatment cannot be made up by assistance from neighbouring cells. In Fig. 2, is a rosette of cells, which had been treated by gamma rays; the cells *D*, are dead, the cells *L*, alive. In the same way the older cells in Fig. 1 had received one dose of x-rays, and were left for some hours, yet they all differ in the manner in which they have responded; the cells *Y* are normal-looking, whereas that at *X* has been badly hit.

In radiated testes of *Abraxas* left for hours or days, the badly stricken spermatocytes drop out and degenerate, leaving gaps in the nest. The survivors go on developing, often quite normally beside the degenerate remains of their less fortunate neighbours.

DISCUSSION

Hegner (1914) shows in his figures of *Leptinotarsa*, clavate spindle bridges, the broad ends of which impinge on the spermatogonial nuclei, but have no connection with heterochromosomes or nucleoli. In a previous paper (Gatenby, 1917) clavate spindle bridges in spermatogonia are drawn, but these do not quite impinge on the nuclei (1917, Pl. 25, Fig. 46, *Euchelia jacobaeae*). In the case of three young spermatocytes of *Euchelia*, there is certainly no connection between the spindle bridges and the nucleoli (1917, Pl. 25, Fig. 45). In recent preparations made by the Nassanow method, it has been found that Golgi bodies exist in the earliest spermatogonial rosettes and in these the spindle bridges certainly do not touch the nuclei, while no nucleoli other than the normal ones can be discovered. The author's previous work on *Euchelia* and *Smerinthus* is also in agreement. The spindle bridges were carefully worked out in a number of species of moths without finding anything like Jan Hirschler's text figure B, and the only conclusion possible is that these moths must differ very much from Jan Hirschler's genera.

On the comparison made by Hirschler between heterochromosomes and the peculiar hemispherical nucleoli, the author is somewhat doubtful but does not care to express a definite opinion.

Hirschler's text figure A seems too definite an arrangement to be artifically produced, and the writer is willing to believe that a trial with Zenker's fluid may show something similar in *Abraxas*. Against this, however, is the fact that Hirschler figures the same thing in text figure B, done by osmic methods, as used by the writer in his collection.

On the question of the origin of Golgi bodies from nuclear sap, the author finds himself in complete disagreement with Jan Hirschler. It will be remembered that Hirschler (1929*b*) has advanced a similar idea in the case of the pentatomid spermatid worked out by him. It is not suggested that mitochondria and Golgi bodies could not originate from nuclear material, but that in the two cases cited by Hirschler, Golgi bodies were already present, before they were supposed by him to arise by some form of condensation.

The response of cells in a nest to radiation, is peculiar and indicates that some of the cells have been affected much more than others. Nor do the unaffected units manage to save their stricken neighbours. Whatever be the exact basis of communication via spindle bridges, between the cells of a nest, it is evident that the radiation injury cannot be repaired by ordinary nutrition as is presumed to be supplied through the channels formed by spindle bridges.

SUMMARY

The spindle bridges of *Abraxas* spermatocytes do not appear to have any relationship with the *de novo* formation of Golgi bodies. No hemispherical nucleoli analogous with the heterochromosomes of

other insects have yet been found in *Abraxas*. The Golgi bodies are present in the smallest spermatogonial rosettes in which the newly-formed spindle bridges are unrelated to the nuclei; in such cells the nucleoli have no connection with the spindle bridges. The spindle bridges, as has been generally concluded, are a means whereby sister cells in a nest are kept at the same state of nutrition. Spermatocytes very badly infected with bacteria, keep step with non-infected sister cells, this, it is concluded, by virtue of the spindle bridges. Radiated testes show nests with dying and apparently undamaged cells side by side. This type of injury appears to be something different from mere inadequacy of nutrition, such as the presence of large numbers of bacteria could bring about.

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